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THE SMC5/6 COMPLEX

LINKING DNA REPLICATION WITH CHROMOSOME SEGREGATION

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The Smc5/6 complex

Linking DNA replication with chromosome segregation

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

In order to faithfully propagate the genetic material from one generation to the next, cells need to properly replicate and segregate their chromosomes. The three well-conserved eukaryotic Structural Maintenance of Chromosomes (SMC) protein complexes, cohesin, condensin and the Smc5/6 complex (Smc5/6) organize chromosomes to ensure that the daughter cells receive a full complement of chromosomes. Cohesin holds sister chromatids, which are the products of replication, together to allow chromosome biorientation prior to segregation. Condensin promotes the condensation of chromosomes to allow them to segregate away from each other during anaphase. The least well-characterized SMC complex, Smc5/6, promotes proper DNA replication, and correct segregation of the ribosomal DNA. Another group of proteins that organizes chromosomes are the topoisomerases. These enzymes cut and paste chromosomes to allow the unwinding of the DNA double helix during replication, and the untangling of chromosomes during segregation. Failure to correctly execute these fundamental processes often leads to cell death. However, it can also lead to cells acquiring the wrong number of chromosomes, *i.e.* aneuploidy, which is a hallmark of cancer cells. Knowledge of how chromosomes are organized and maintained is therefore important not only to understand the basic principles of life, but also to understand cancerous cells.

With the projects presented in this thesis, we aimed to extend our knowledge about the functions of Smc5/6 and topoisomerases during DNA replication and chromosome segregation, using the model organism *Saccharomyces cerevisiae* (*S. cerevisiae*). Since the SMC complexes perform their functions by directly associating with chromosomes, an important focus of our studies has been to characterize the chromosomal association pattern of Smc5/6 in detail, in order to reveal new clues about its functions. The main findings of the four projects are introduced below.

In **Paper I**, we presented new functions of Smc5/6 and type I topoisomerases in the timely replication of long *S. cerevisiae* chromosomes. We also showed that the chromosomal association of Smc5/6 is regulated by chromosome length and topoisomerase II. The data allowed us to propose a model in which Smc5/6 promotes replication by stimulating fork rotation to reduce topological stress ahead of the fork.

In **Paper II**, we showed that Smc5/6 requires sister chromatids to be held together in order to associate with chromosomes. Smc5/6 was also shown to promote correct segregation of short entangled chromosomes. Our extensive characterization of the chromosomal

association of Smc5/6 led us to the hypothesis that Smc5/6 associates to chromosomal loci where the sister chromatids are entangled, and that topological stress during replication affect the level of chromosome entanglement.

In **Paper III**, we created a hard-to-replicate region of DNA by artificially inducing high convergent RNA polymerase II-driven transcription. This caused the replication fork to pause, which was dependent on the highly expressed gene that opposed the direction of replication. The paused fork was assisted past this obstacle by the Rrm3 helicase. In addition, Smc5/6 associated to chromatin behind the paused fork, where it remained also after replication. Our results strengthened the hypothesis that topological stress is a factor that contributes to the recruitment of Smc5/6 to chromosomes.

In **Paper IV**, we dissected the role of the Nse5 subunit of Smc5/6 during replication stress induced by hydroxyurea, which inhibits the production of nucleotides. We showed that Nse5 is required for the sumoylation of Smc5, and the recruitment of the complex to stalled forks. The results also indicated that the former of these functions is dispensable, while the latter is important, for Smc5/6 to stabilize stalled replication forks and prevent aberrant recombination at these forks.

The results of this thesis increase our understanding of how chromosomes are replicated and segregated, and highlight the importance of analyzing the topological status of chromosomes to fully understand the processes that maintain genome stability.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following articles and manuscript, which are referred to in the text by their Roman numerals.

- I. Kegel A, Betts-Lindroos H, Kanno T, **Jeppsson K**, Ström L, Katou Y, Itoh T, Shirahige K, Sjögren C.
Chromosome length influences replication-induced topological stress.
Nature. 2011 Mar 17;471(7338):392-6.
- II. **Jeppsson K**, Carlborg KK, Nakato R, Berta DG, Lilienthal I, Kanno T, Lindqvist A, Brink MC, Dantuma NP, Katou Y, Shirahige K, Sjögren C.
The chromosomal association of the Smc5/6 complex depends on cohesion and predicts the level of sister chromatid entanglement.
PLoS Genet. 2014, 10(10): e1004680.
- III. **Jeppsson K**, Kegel A, Shirahige K and Sjögren C.
Transcription-dependent replication fork pausing attracts the Smc5/6 complex to chromosomes.
Manuscript
- IV. Bustard DE, Menolfi D, **Jeppsson K**, Ball LG, Dewey SC, Shirahige K, Sjögren C, Branzei D, Cobb JA.
During replication stress, non-SMC element 5 (NSE5) is required for Smc5/6 protein complex functionality at stalled forks.
J Biol Chem. 2012, 287, 11374-11383.

RELATED PUBLICATION, NOT INCLUDED IN THE THESIS

Jeppsson K, Kanno T, Shirahige K, Sjögren C.
The maintenance of chromosome structure: positioning and functioning of SMC complexes.
Nat Rev Mol Cell Biol. 2014 Sep;15(9):601-14.

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LIST OF ABBREVIATIONS

SMC	Structural Maintenance of Chromosomes
ORC	Origin recognition complex
ARS	Autonomously replicating sequence
Pre-RC	Pre-replication complex
CDK	Cyclin-dependent kinase
dNTP	Deoxynucleoside triphosphate
SCI	Sister chromatid intertwining
Top1	Topoisomerase 1
Top2	Topoisomerase 2
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
ChIP	Chromatin immunoprecipitation
qPCR	Quantitative polymerase chain reaction
GFP	Green fluorescent protein
BrdU	Bromodeoxyuridine
PFGE	Pulse-field gel electrophoresis
FACS	Fluorescence-activated cell sorting
HU	Hydroxyurea
RFB	Replication fork barrier
rDNA	Ribosomal DNA
tRNA	Transfer RNA
SUMO	Small Ubiquitin-like Modifier
RNAPII	RNA polymerase II
RNAPIII	RNA polymerase III
bp, kb	Base pairs, kilobase pairs

INTRODUCTION

Chromosomes are composed of long DNA double helices. To proliferate, cells have to perform the formidable tasks of unwinding and replicating these long molecules accurately, and thereafter condense and properly segregate them into daughter cells. To avoid overwinding of the DNA helix during replication and tangling of the sister chromatids that can prevent chromosome segregation, cells rely on enzymes called topoisomerases. These enzymes transiently break chromosomes to release topological stress, and to resolve entanglements. To further organize chromosomes and maintain genome stability, the three eukaryotic Structural Maintenance of Chromosomes (SMC) protein complexes perform fundamental tasks. Cohesin holds sister chromatids together from the time they are formed by replication until they are segregated during anaphase. This is important to ensure bipolar attachment of chromosomes in metaphase. Condensin helps to compact chromosomes prior to anaphase to promote their proper segregation. The third SMC complex, the Smc5/6 complex (hereafter referred to as Smc5/6), is less well studied. At the start of this thesis, Smc5/6 had been shown to perform functions during DNA repair by homologous recombination and to promote segregation of the ribosomal DNA (rDNA). To learn more about this elusive complex, the main focus of the thesis was to explore the functions and chromosomal association of Smc5/6, and how this influences, and is influenced by, the topological status of chromosomes.

SPECIFIC AIMS OF THIS THESIS

In **Paper I**, to investigate the intriguing finding that Smc5/6 associates with chromosomes in a chromosome-length dependent manner (Lindroos et al., 2006). In addition, the aim was to elucidate Smc5/6 functions and its relationship to topoisomerases during DNA replication.

In **Paper II**, to explore the hypothesis, proposed in **Paper I**, that Smc5/6 chromosomal association is triggered by sister chromatid intertwinings (SCIs), and to investigate the function of Smc5/6 in the segregation of entangled chromosomes.

In **Paper III**, to further investigate the hypothesis that topological stress is a factor that determines the chromosomal association of Smc5/6.

In **Paper IV**, to investigate the functions of the Nse5 subunit of Smc5/6 during replication stress.

MAINTENANCE OF GENOME STABILITY

DNA replication and chromosome segregation are central for cell proliferation. To allow the faithful transmission of genetic material to daughter cells, chromosomes need to be accurately replicated. The products of DNA replication, sister chromatids, also require to be held together until mitosis, and then fully untangled, to ensure that daughter cells receive an equal set of chromosomes. In addition, any potential damage to the DNA needs to be repaired accurately to maintain the stability of the genome. One event that can create DNA damage is if the replication machinery encounters obstacles. To counteract breakage, and ensure the proper resumption of replication after the obstacle has been cleared, replication forks need to be stabilized. In the sections below, these processes are described with a focus towards serving as an introduction to the papers and discussion parts of the thesis.

DNA REPLICATION

DNA replication is a highly controlled process that allows the duplication of the genome in a rapid and accurate manner. In eukaryotes, replication is started at multiple origins on each chromosome to allow swift replication completion of the large genomes. At an origin, two replication machineries (replisomes) are established, which at the time of origin firing move away from each other in a bidirectional manner. This creates a replication bubble with a replication fork at either end, *i.e.* the Y-shaped structure where the parental DNA molecule converts into the two newly formed sister chromatids. The DNA molecule is replicated in a semi-conservative manner, meaning that the parental DNA helix is unwound and new complementary strands are synthesized. This results in the formation of identical sister chromatids, which each are composed of one DNA strand from the parental DNA double helix and one newly synthesized strand. Since DNA strands can only be built in 5' to 3' direction, and DNA double helices are composed of two antiparallel strands, the replisome needs to synthesize one of the new strands in the direction of fork movement (the leading strand), and the other one in the direction opposite to the fork movement (the lagging strand) (Figure 1). The leading strand is therefore synthesized as a single molecule, whereas the lagging strand is continuously re-primed by an RNA primase and synthesized in short pieces, known as Okazaki fragments. The RNA primers are subsequently removed from the Okazaki fragments and replaced with DNA, and lastly the fragments are ligated together.

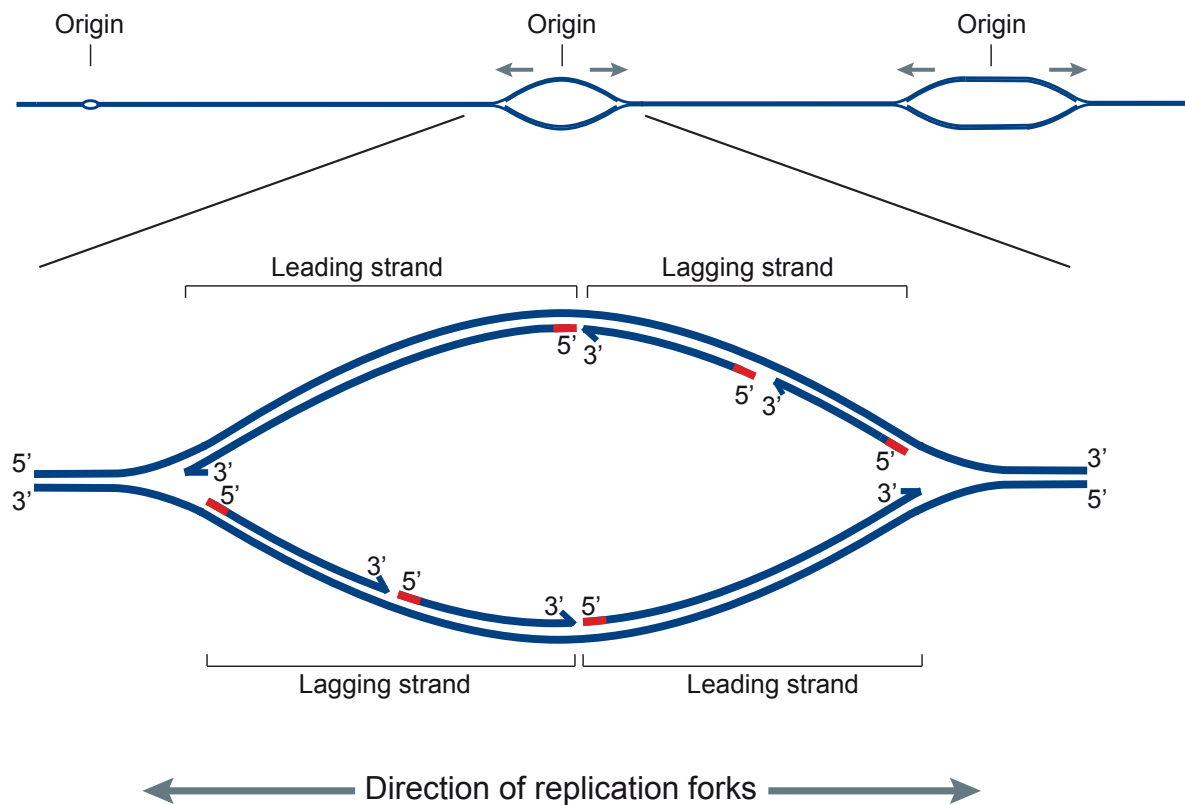


Figure 1. DNA replication proceeds bidirectionally from origins

DNA replication initiates from multiple origins on eukaryotic chromosomes (top panel). Some origins are fired early in S-phase and others later, *e.g.* the rightmost origin has fired early, whereas the leftmost origin has not yet fired. A close-up of a replication bubble is displayed in the lower panel. DNA is synthesized in 5'-3' direction, which leads to that the lagging strand is replicated discontinuously in shorter Okazaki fragments. The red parts at the 5' end of newly synthesized strand denote RNA primers, which are later removed and replaced by DNA.

A strict temporal regulation of replication initiation ensures that all chromosomal loci replicates precisely once per cell cycle. In eukaryotes, the origin recognition complex (ORC) binds to replication origins (Bell and Stillman, 1992). Origins in *Saccharomyces cerevisiae* (*S. cerevisiae*) are defined by specific sequences called autonomously replicating sequences (ARS), which received their names because they were originally characterized to support plasmid maintenance (Newlon, 1988). At each origin, ORC, together with the help of the licensing factors Cdc6 and Cdt1, loads two copies of the inactive hexameric helicase Mcm2-7, in a reaction called origin licensing (Evrin et al., 2009; Remus et al., 2009). These factors make up the pre-replication complex (pre-RC), and their loading onto chromatin is restricted to late mitosis/early G1 by the degradation of Cdt1 in S-phase, and by cyclin-dependent kinase (CDK) activity. This prevents re-replication by ensuring that new pre-RC cannot be formed during S-phase. The pre-RC then recruits additional factors including Cdc45 and GINS to form the pre-initiation complex (Moyer et al., 2006; Tercero et al., 2000). Lastly,

CMG (Cdc45, Mcm2-7 and GINS) is activated in S-phase by CDK and Dbf4-dependent kinase (DDK) in a reaction that was recently reconstituted *in vitro* (Yeeles et al., 2015).

The termination of replication in eukaryotes is considerably less well characterized than the initiation. One reason for this is the fact that replication termination was found to occur in wide regions, instead of at particular loci (Greenfeder and Newlon, 1992b), which makes it more difficult to analyze. In this study, the deletion of an origin was shown to alter the position of the termination region, which suggested that termination sites are not predetermined by specific sequences. Later, a genome-wide study of termination between early firing origins confirmed that termination occurs in wide regions, but suggested that these regions contain replication fork pausing elements, such as highly transcribed genes or centromeres (Fachinetti et al., 2010). These elements were suggested to pause one of the forks until the converging fork arrives. However, this idea was challenged by a study analyzing replication termination by sequencing Okazaki fragments (McGuffee et al., 2013). Their data argued against that replication forks were paused at specific sites to induce termination, by showing that termination generally occurs midway between two origins, if they are fired at the same time. Recently, two pioneering studies showed that ubiquitylation of the replicative helicase subunit Mcm7 during the final stages of replication, promotes the disassembly of the terminated replisome (Maric et al., 2014; Moreno et al., 2014). These findings indicate that termination of replication could be as well controlled as initiation.

Replication fork pausing

Replication forks can encounter both natural and abnormal obstacles that need to be overcome to complete the proper duplication of chromosomes. A well-described natural replication obstacle exists in the rDNA in *S. cerevisiae*. Here, the replication fork barrier (RFB) pauses one of the replication forks to ensure that replication only proceeds codirectionally with rDNA transcription (Brewer and Fangman, 1988). Fork pausing at the RFB is dependent on the Fob1 protein (Kobayashi and Horiuchi, 1996), which binds tightly to the RFB sequence (Kobayashi, 2003). Other natural fork obstacles include centromeres (Greenfeder and Newlon, 1992a), inactive origins (Ivessa et al., 2003) and RNA polymerase III (RNAPIII)-transcribed genes opposing the direction of replication (Deshpande and Newlon, 1996). The replication fork is assisted by the Rrm3 helicase, known as sweepase, past obstacles consisting of non-histone proteins bound tightly to DNA (Ivessa et al., 2003; Ivessa et al., 2000). Highly expressed RNA polymerase II (RNAPII)-transcribed genes can

also pause replication forks, however it is debated if such pausing is restricted to genes oriented against the incoming fork, and if these paused forks are helped by Rrm3 (Azvolinsky et al., 2009; Prado and Aguilera, 2005).

Another form of natural impediments to fork progression is high levels of topological stress, which can be formed ahead of translocating replisomes and transcription machineries (see below). High levels of topological stress can cause complete fork pausing, since topoisomerases are required for replication progression. However, it is difficult to distinguish between the contribution of topological stress, as opposed to direct collision between replisome and RNA polymerase, to fork pausing caused by transcription opposing the replication direction. An elegant study provided evidence that replication fork reversal, which can occur at stalled replication forks in the absence of a functional checkpoint, was due to the build-up of high topological stress (Bermejo et al., 2011). In checkpoint mutants, fork reversal occurred when the replication fork encountered a transcription unit, at which the process of transcription was coupled to mRNA export by attachment of the chromatin to nuclear pore complexes, known as gene gating. Such RNA-mediated anchoring of chromatin has the potential to serve as a barrier to the topological stress ahead of the replication fork. The authors showed that by creating a DNA break in the vicinity of the replication fork, which would release any topological stress, fork reversal was avoided.

Formation of DNA loops at transcribed genes creates another type of topological structure that could be the cause for the suggested fork pausing at highly expressed genes that are transcribed in the same direction as replication (Azvolinsky et al., 2009). This has been suggested occur by looping that places the terminator region next to the promoter region (Ansari and Hampsey, 2005), mediated by topoisomerase 2 (Top2) and Hmo1 (Bermejo et al., 2009).

Replication progression can also be halted by the presence of chemical compounds that cause alkylation of the DNA template, or inhibits the production of nucleotides, such as methyl methanesulfonate (MMS) and hydroxyurea (HU), respectively. When replication forks stop due to obstacles, which are not easy to overcome, such as chemically induced obstacles, they are often referred to as “stalled” forks. Related to this thesis, HU inhibits the enzyme ribonucleotide reductase, which normally functions in the production of the building blocks of DNA, *i.e.* deoxynucleoside triphosphates (dNTPs). The presence of HU therefore inhibits the accumulation of dNTPs that occurs in unchallenged cells in the beginning of S-phase (Chabes et al., 2003; Koc et al., 2004). In the presence of HU, early origins fire, but

then replication progression comes to a quick halt close to these origins, when the basal levels of dNTPs are consumed. The slowdown of replication forks exposes single-stranded DNA that leads to recruitment of Mec1, a checkpoint kinase (Sogo et al., 2002). Mec1 then activates Rad53, which prevents firing of late origins and stabilizes the replication fork, to prevent them from collapsing, *i.e.* falling off chromatin (Lopes et al., 2001).

CHROMOSOME SEGREGATION

Chromosome segregation allows the precise division of the genetic material into daughter cells. For the cell to distinguish which DNA molecules are going to be separated from each other, the sister chromatids are held together from the time they are formed in S-phase, until anaphase, when chromosome segregation occurs. The process of holding sister chromatids together, known as sister chromatid cohesion, is dependent on the SMC complex cohesin and is described in detail below. In addition, a force is required to pull the sister chromatids apart when sister chromatid cohesion is dissolved. This force is provided by the spindle apparatus, which attaches microtubules to a protein structure formed at the centromere of chromosomes, called the kinetochore. The correct attachment of the spindle to the kinetochore is monitored by the spindle assembly checkpoint (SAC), which delays anaphase onset until all kinetochores are attached to microtubules. An important note for this thesis is that in *S. cerevisiae* microtubules are attached to kinetochores throughout the cell cycle, except for a brief period during S-phase when the centromeric regions are replicated and kinetochores are transiently disassembled (Kitamura et al., 2007).

In anaphase, when all kinetochores have been attached and the SAC has been silenced, cohesin is cleaved by the protease separase (Uhlmann et al., 1999). Prior to anaphase, separase is kept inactive by binding to securin (Ciosk et al., 1998). At anaphase onset, the anaphase promoting complex, together with its coactivator Cdc20, trigger degradation of securin, which activates separase to cleave cohesin. This allows sister chromatids to separate from each other and segregate to opposite poles. The function of Top2, the enzyme that resolves entangled chromosomes, is essential in mitosis to avoid chromosome breakage (Holm et al., 1985; Spell and Holm, 1994). This suggests that any remaining entanglements between sister chromatids must be resolved at mitosis to allow correct segregation (see details below).

DNA TOPOLOGY

Chromosomes carry the genetic information as long double helices. The DNA double helix, consisting of two non-covalently bound single strands, completes a full right-handed turn around its helical axis approximately every 10.5 base pairs (bp) in its relaxed form. Due to the long length of chromosomes, the unwinding of DNA double helices to allow semi-conservative replication during S-phase appears as a challenging task. In addition, the chromosomes need to be separated without tangling to avoid breakage during segregation. The study of DNA topology concerns the shape and path of DNA strands in space, and aims to understand the transitions of DNA molecules during replication and chromosome segregation (Bates and Maxwell, 2005; Wang, 2002).

An important concept of DNA topology is that the DNA helix can become supercoiled. Twisting one end of a relaxed DNA molecule, while hindering the free rotation of the other end, creates topological stress in the molecule. If the molecule is being overwound, the number of full turns (twists) of the helix increases. Eventually the torsional

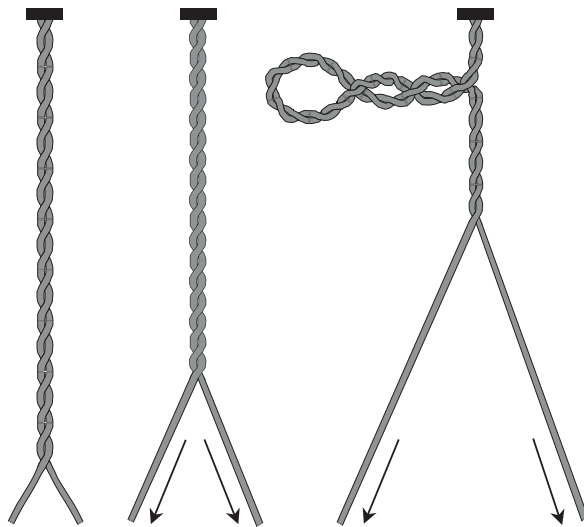


Figure 2. Increased twist causes supercoiling

Pulling apart the strands of a twisted rope leads to increased twisting ahead of the opening, if the distal end is prevented from rotating. Eventually the increased twist leads to that the rope coils upon itself.

stress of the helix will cause it to coil onto itself, *i.e.* become supercoiled. Overwinding of the helix creates positive supercoils and conversely underwinding creates negative supercoils. A commonly used analogy of twist-induced supercoiling is if the intertwined strands of a rope are pulled apart, which causes the rope to coil on itself (Figure 2). To allow the processes of DNA replication and chromosome segregation, a special class of enzymes called topoisomerases cut and re-ligate DNA strands to resolve topological stress.

In regards to this thesis, we use the words superhelical tension, superhelical stress and topological stress interchangeably, to refer to the accumulation of topological structures, *e.g.* twists and supercoils, which can be created during the unwinding of the DNA helix during replication and transcription.

TOPOISOMERASES

Topoisomerases are enzymes that regulate the over- and underwinding, or entanglement, of DNA molecules. They do so by creating transient DNA breaks in the phosphate backbone of the molecules. There are two types of topoisomerases, type I and type II. Type I topoisomerases cleave a single DNA strand of the double helix, and rotate the broken strand around the intact strand. This allows the resolution of positive and negative supercoils. The type I topoisomerases are further subdivided into either type IA or type IB. After the single strand cleavage by a type IA topoisomerase, it remains covalently attached to the created 5' end of the DNA molecule. The free 3' end is then moved around the intact DNA strand by non-covalent attachment to the topoisomerase, before the single strand break is religated (Wang, 2002). Type IB topoisomerase instead remains covalently bound to the 3' end of the broken DNA strand, and the created 5' end of is then allowed to rotate freely around the intact strand. This means that type IA topoisomerases perform a stepwise relaxation, whereas type IB can release more topological stress in one reaction. Type II topoisomerases function as dimers and cleave both strands of a DNA molecule. They then pass an intact DNA molecule through the transient opening, before resealing the double strand break. By this mechanism, type II topoisomerases can resolve supercoils, as well as SCIs.

The *S. cerevisiae* genome encodes for three topoisomerases, Top1 (type IB), Top2 (type II) and Top3 (type IA). Top1 is non-essential in *S. cerevisiae* (Goto and Wang, 1985), unlike in more complex eukaryotes (Lee et al., 1993; Morham et al., 1996). Top2, on the other hand, is essential in *S. cerevisiae* (Goto and Wang, 1985). The essential function of Top2 is performed in mitosis (Holm et al., 1985). In the absence of Top2 in anaphase, chromosomes missegregate and break in a length-dependent manner, with longer chromosomes breaking more frequently, likely due to unresolved SCIs (Spell and Holm, 1994). In the absence of Top3, *S. cerevisiae* cells grow slowly and show a hyper-recombinogenic phenotype (Wallis et al., 1989). These phenotypes are suppressed by deletion of Sgs1, an *E. coli* RecQ helicase homolog (Gangloff et al., 1994), which led to the hypothesis that Top3 resolves structures created by Sgs1.

TOPOLOGICAL TRANSITIONS DURING TRANSCRIPTION

As the RNA polymerase locally unwinds the DNA helix and rapidly translocates along a gene, the DNA becomes overwound (positively supercoiled) ahead and underwound (negatively supercoiled) behind of the transcription unit (Liu and Wang, 1987). This is referred to as the twin-model of transcriptional supercoiling. In *S. cerevisiae*, Top1 and Top2 are responsible for relaxing both negative and positive supercoils during transcription (Figure 3A).

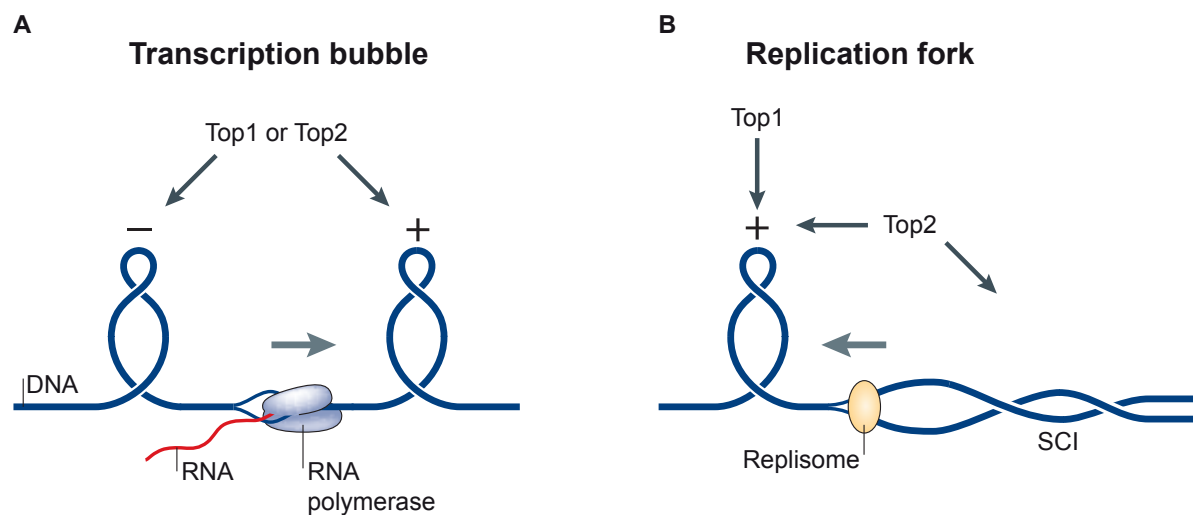


Figure 3. DNA topology during transcription and replication

(A) Positive (+) supercoils accumulate ahead of a translocating RNA polymerase, while negative (-) supercoils accumulate behind. In *S. cerevisiae*, both positive and negative supercoils can be resolved by Top1 and Top2. **(B)** Positive supercoils also accumulate ahead of an advancing replisome, which can be resolved by Top1 and Top2. If the replication fork rotates with the turn of the parental DNA helix, positive supercoils ahead of the fork can be avoided, but instead SCIs accumulate behind the replication fork. These SCIs require Top2 for their enzymatic resolution. Adapted from Jeppsson et al., 2014.

S. cerevisiae cells can support proper transcription of most genes in the absence of either Top1 or Top2 functions. In the absence of both Top1 and Top2, transcription of the rDNA is largely inhibited (Brill et al., 1987). However, transcription is not strongly reduced in the rest of the genome, which is likely due to that negative and positive topological stress can cancel each other out (Stupina and Wang, 2004). This is supported by the finding that if the *Escherichia coli* (*E. coli*) topoisomerase I, which only relaxes negative supercoils, is expressed in *S. cerevisiae top1 top2* double mutant, global RNA synthesis is strongly reduced (Gartenberg and Wang, 1992). This suggests that the accumulation of positive supercoiling can block transcription throughout the genome.

Using more sensitive techniques, differences of removing Top1 or Top2 functions in *S. cerevisiae* could be detected. In the transcription of the rDNA, *top2* mutant cells displayed slower transcription elongation, indicative of that Top2 is the main topoisomerase that removes positive supercoils in this region (French et al., 2011). *top1* mutant cells on the other hand accumulated negative supercoils, highlighting the importance of this topoisomerase in the resolution of supercoils behind the transcription machineries in the rDNA. Top2 was also recently shown to have a specific role for the proper transcription of long (>3 kilobase pairs (kb)) *S. cerevisiae* genes throughout the genome (Joshi et al., 2012). The authors speculated that in long genes, topological stress ahead of the transcription unit was more often converted into positive supercoils, which Top2's double strand passing mechanism is more efficient in resolving than Top1's nicking mechanism. In short genes on the other hand, the topological stress ahead of the transcription machinery more often might remain as increased twist or overwound DNA, which Top1 is fully capable of resolving.

Related to this thesis, a study showed that genes situated within 100 kb of a telomere gradually escaped from the transcription stalling caused by expressing *E. coli* topoisomerase I in *top1 top2* mutant cells (Joshi et al., 2010). These results strongly indicated that topological stress in the form of positive supercoils or overwound DNA can dissipate over *S. cerevisiae* chromosome ends. Another important point concerning topology during transcription related to this thesis, is that re-orienting a pair of highly expressed RNAPII genes from a tandem to a convergent orientation, did not reduce their transcription levels (Prescott and Proudfoot, 2002). However, such convergently oriented RNAPII genes are highly dependent on both Top1 and Top2 for their proper transcription (Garcia-Rubio and Aguilera, 2012). This is true also if the transcript lengths of the convergently oriented genes is shorter than 3 kb. These findings indicate that high levels of topological stress accumulate at closely situated convergently oriented highly expressed genes.

TOPOLOGICAL TRANSITIONS DURING REPLICATION

The unwinding of the parental DNA helix by the replicative helicase during replication causes the region ahead of the replication fork to become positively supercoiled (Figure 3B). In *S. cerevisiae* cells, Top1 or Top2 can resolve this topological stress in order to allow replication fork progression. In the absence of both Top1 and Top2 functions, replication stalls a few kb from origins (Brill et al., 1987; Kim and Wang, 1989). The fact that *top1 top2* double mutants cannot replicate their chromosomes shows that Top3 is unable to support

proper replication progression. Top3 role during unchallenged replication remains unknown, but it has been suggested to resolve structures formed between two converging replication forks (Mankouri and Hickson, 2007).

Another way to diminish positive supercoils ahead of the fork, and promote fork progression, is if the replication fork rotates with the turn of the parental helix. This would then channel positive supercoils ahead of the replication fork into SCIs behind the fork. Fork rotation was suggested to occur mainly during replication termination when the length of the region between the two converging forks becomes too short for topoisomerases to act on (Champoux, 2001; Sundin and Varshavsky, 1980). The SCIs formed during replication need to be resolved by Top2 to allow proper chromosome segregation in anaphase (Spell and Holm, 1994).

STRUCTURAL MAINTENANCE OF CHROMOSOMES

In eukaryotes, the well-conserved SMC complexes, cohesin, condensin and Smc5/6 perform fundamental processes to organize chromosomes and maintain genome stability. Cohesin holds newly replicated sister chromatids together to ensure bipolar attachment of chromosomes and accurate segregation. Condensin is required for chromosome condensation, which is important to allow complete chromosome separation during mitosis. Smc5/6 is less well characterized than the other two complexes, but has been shown to promote DNA repair by homologous recombination, timely DNA replication, and segregation of the rDNA. Condensin will not be discussed in detail below, since it lies outside the scope of thesis.

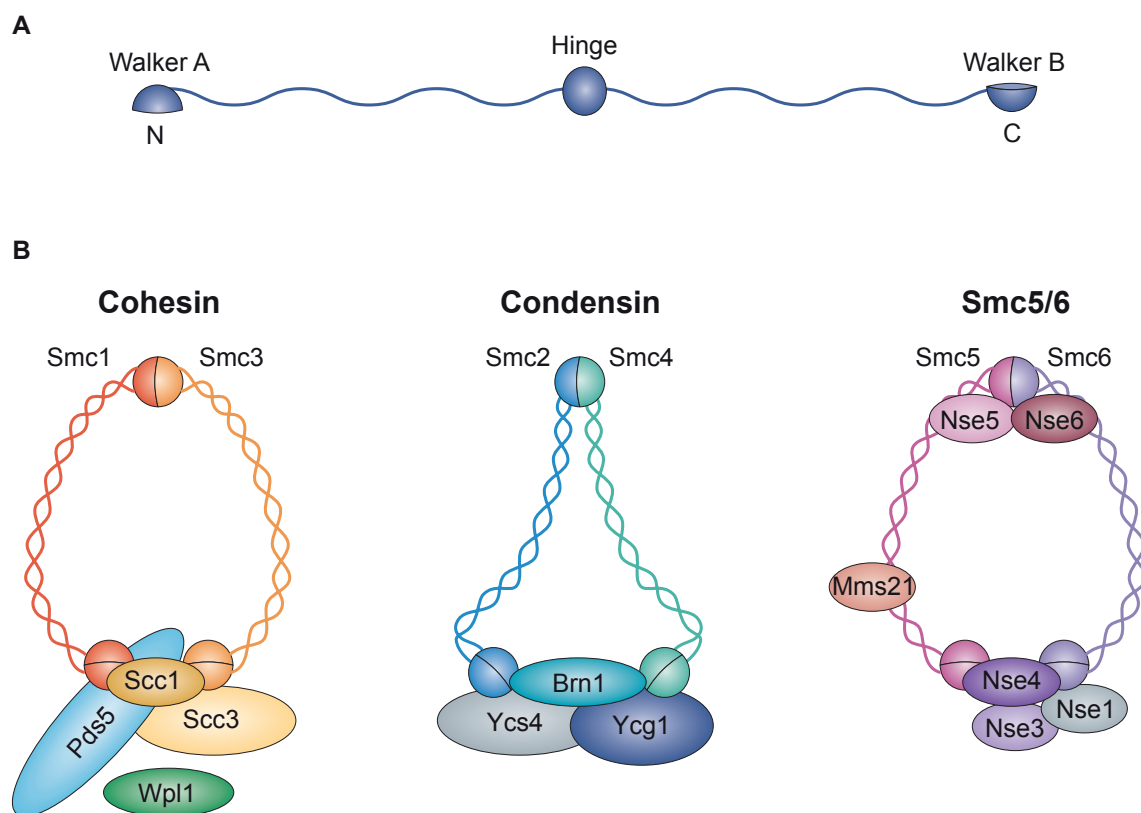


Figure 4. Structure and composition of SMC complexes

(A) Domains of an unfolded SMC protein. The SMC protein then folds back on itself at the hinge domain, which brings the Walker A and Walker B motives together to form the head domain. (B) Structure and composition of the three SMC complexes in *S. cerevisiae*. Adapted from Jeppsson *et al.*, 2014.

STRUCTURE AND COMPOSITION OF SMC COMPLEXES

The eukaryotic SMC complexes are built around a core of a unique heterodimer of SMC proteins. SMC proteins are 1000-1500 amino acids in length and have a characteristic structure. In the center of an SMC protein there is a hinge domain, and at both the N- and C-termini there are globular domains containing Walker A and Walker B motifs, respectively

(Figure 4A). The protein folds back on itself at the hinge domain, which brings the N- and C-termini together to form a functional ATPase domain. The two regions between the hinge domain and each terminus interact with each other to form a long anti-parallel coiled-coil structure. The SMC proteins then dimerize in specific pairs for each SMC complex, by interacting at the hinge domains to form V-shaped heterodimers. In addition to the SMC proteins, each complex contains a number of non-SMC subunits. One of these subunits is a member of the kleisin protein family, which bridges the two ATPase-containing head domains of the SMC heterodimer, and thereby transforms the V-shaped dimer into the characteristic ring-shaped SMC complex structure (Figure 4B) (Jeppsson et al., 2014). The subunits of cohesin and Smc5/6 are presented in more detail below.

Cohesin composition

The four canonical subunits of cohesin are Smc1 and Smc3 that make up the core heterodimer, the kleisin subunit Scc1, and Scc3 (Guacci et al., 1997; Michaelis et al., 1997; Toth et al., 1999). The head domains of the Smc1-Smc3 heterodimer are bridged by Scc1, which creates a well-characterized tripartite ring structure (Haering et al., 2008; Haering et al., 2002). In addition to these four subunits there are cohesin-interacting proteins important for its function. One of them is Pds5, which binds to cohesin through Scc1 (Hartman et al., 2000; Panizza et al., 2000). Another cohesin-interacting protein is Wapl (Kueng et al., 2006), which binds to Pds5. However unlike Pds5, Wapl was shown to interact with cohesin in a substoichiometric manner, showing that cohesin complexes do not always contain Wapl (Chan et al., 2012). In human cells there is also a protein called sororin, which interacts with cohesin and is needed for its function (Schmitz et al., 2007). However, sororin does not associate with cohesin throughout the cell cycle, instead it has been suggested to interact with cohesin only when the complex holds sister chromatids together (Nishiyama et al., 2010).

Smc5/6 composition

The core heterodimer of Smc5/6 is, as the name implies, composed of the SMC proteins Smc5 and Smc6 (Fousteri and Lehmann, 2000). In addition, the complex contains six other subunits, out of which two have only been found in yeast. Nse1, Nse3, and the kleisin-like protein Nse4 form a subcomplex, which bridges the head domains of Smc5 and Smc6 (Palecek et al., 2006). Mms21 is a small ubiquitin-like modifier (SUMO) E3 ligase that binds

to the coiled-coil arm of Smc5 (Zhao and Blobel, 2005). Both *S. cerevisiae* and *Schizosaccharomyces pombe* (*S. pombe*) have the additional subunits Nse5 and Nse6 (Pebernard et al., 2006; Zhao and Blobel, 2005). However, although they share the same names, they are not conserved on the sequence level between the two yeast species. They also associate with different parts of the remaining complex, since Nse5 and Nse6 in *S. cerevisiae* associate with the hinge domains of Smc5 and Smc6 (Duan et al., 2009), whereas *S. pombe* Nse5 and Nse6 associate with the head domains of the heterodimer (Palecek et al., 2006).

FUNCTIONS OF SMC COMPLEXES

The SMC complexes act as functional units, with little evidence that individual subunits can perform individual tasks. *In vitro* studies have shown that cohesin and condensin can link two DNA duplexes together in an ATP-dependent manner. Cohesin was shown to promote intermolecular DNA linking, while condensin promoted intramolecular DNA linking (Kimura et al., 1999; Losada and Hirano, 2001). In addition, unpublished data from the Sjögren lab have shown that Smc5/6, similarly to cohesin can link two different DNA molecules together in an ATP-dependent manner (Kanno and Sjögren, unpublished). Potentially, the basal mechanism of the *in vivo* functions of SMC complexes is to bridge two DNA loci. This could account for cohesin's and condensin's functions in sister chromatid cohesion and condensation. However, since SMC complexes affect a wide variety of chromosomal processes, the regulation or downstream effects of such bridging functions most likely are extensive. Smc5/6 also includes a SUMO-ligase, whose targets can be involved in many processes, which are unrelated to DNA bridging. In the two sections below, the *in vivo* functions of cohesin and Smc5/6, which relate to this thesis, are introduced.

Cohesin functions

The main function of cohesin is sister chromatid cohesion (Guacci et al., 1997; Michaelis et al., 1997). Through the action of holding sister chromatids together, cohesin counteracts the pulling forces of the spindle apparatus and thereby promotes chromosome biorientation (Tanaka et al., 2000). The mechanism by which cohesin holds sister chromatids together has been well studied. Cohesin forms a ring structure *in vivo* (Gruber et al., 2003) and is capable of topologically entrap DNA molecules within its ring (Haering et al., 2008; Murayama and Uhlmann, 2014). Artificial cleavage of the ring structure abolishes cohesion between sister

chromatids (Gruber et al., 2003; Haering et al., 2008). Since cohesin assembles as a complex before associating with chromatin (Ciosk et al., 2000), the ring structure requires opening to allow topological entrapment of DNA. This has been proposed to occur by the transient opening of the Smc1-Smc3 hinge interface (Gruber et al., 2006). The Smc1-Smc3 interface was therefore termed cohesin's "entry gate". Conversely, to allow the dynamic interaction of cohesin with chromosomes, and the cleavage-independent removal of cohesin from chromosome arms in prophase (see below), DNA molecules should also be able to exit from the cohesin ring. This was proposed to occur, not through the Smc1-Smc3 interface, but instead through the Smc3-Scc1 interface (Buheitel and Stemmann, 2013; Chan et al., 2012; Huis in 't Veld et al., 2014). Together these findings support a ring model where cohesin's topological entrapment of sister chromatids is how the spindle force is counteracted. The simplest form of a ring model is the "one-ring" or "embracement" model in which a single cohesin complex encircles the two sister chromatids (Haering et al., 2002). However, if two DNA molecules can actually be entrapped within a single ring remains unknown. There are also alternative ring models, such as the "handcuff"-model (Huang et al., 2005; Zhang et al., 2008), in which two cohesin complexes interact, each with its own entrapped sister chromatid.

Cohesin also protects SCIs from resolution by Top2 on long (26 kb) plasmids in G2/M-phase (Farcas et al., 2011). This is however not the case on shorter (14 kb) plasmids (Koshland and Hartwell, 1987). Importantly, results from the study of longer plasmids suggested that cohesin was able to hold plasmids together even if they were not intertwined. The fact that Top2 is essential in mitosis (Holm et al., 1985), and that chromosomes missegregate when Top2 is inactivated solely during mitosis (Uemura et al., 1987), suggests that SCIs are also protected from resolution on linear chromosomes until cohesin is removed.

In addition to sister chromatid cohesion, cohesin promotes condensation of the rDNA in *S. cerevisiae* (Guacci et al., 1997). In mouse embryonic fibroblasts, depletion of Wapl, which counteracts cohesin's stable association with chromosomes, causes condensation of interphase chromosomes (Tedeschi et al., 2013). Similarly, deletion of Wapl in *S. cerevisiae* cause increased condensation the right arm of chromosomes 12, where the rDNA array is located (Lopez-Serra et al., 2013). This suggests that a balanced and dynamic association of cohesin with chromosomes is required to properly organize chromosomes.

Cohesin also affects transcription in human cells, and has been suggested to perform its gene regulatory function by mediating long-range chromosomal interactions in *cis* between enhancers and promoters (Hadjur et al., 2009; Kagey et al., 2010).

Smc5/6 functions

Mutations in Smc5/6 subunits cause cells to be hypersensitive to DNA damaging agents such as MMS, ultraviolet light (UV) and ionizing irradiation, and also to nucleotide-depletion by HU (Andrews et al., 2005; Lehmann et al., 1995; Pebernard et al., 2006; Verkade et al., 1999). Epistasis analyses have shown that Smc5/6 functions in DNA repair by homologous recombination (Andrews et al., 2005; Torres-Rosell et al., 2005; Verkade et al., 1999). A function in homologous recombination is supported by the observation that homologous recombination-dependent structures accumulate at damaged replication forks in Smc5/6 mutants (Ampatzidou et al., 2006; Brnzei et al., 2006). Smc5/6 has been suggested both to recruit recombination proteins, and later to promote the resolution of recombination intermediates at the damaged forks (Irmisch et al., 2009). Smc5/6 also function in homologous recombination during meiosis, by preventing and resolving aberrant recombination intermediates (Copsey et al., 2013; Lilienthal et al., 2013; Xaver et al., 2013).

Unlike most other proteins involved in homologous recombination in yeast, Smc5/6 is also essential in unchallenged cells (Lehmann et al., 1995). This essential function remains largely elusive. Unchallenged *S. cerevisiae* cells fail to properly segregate the rDNA in Smc5/6 mutants (Torres-Rosell et al., 2005). This was suggested to be due to that Smc5/6 mutants failed to complete the replication of the rDNA before entering anaphase (Torres-Rosell et al., 2007). However, deleting the endogenous rDNA array and instead placing a single rDNA unit on a multicopy plasmid, which simplifies its segregation, did not improve the growth of Smc5/6 mutants (Torres-Rosell et al., 2005). This shows that there is another essential function performed by Smc5/6, other than promoting segregation of the rDNA.

In human cells, depletion of both Smc5 and Smc6 resulted in chromosomes displaying an abnormal structure in metaphase, and aberrant linkages between sister chromatids during anaphase (Gallego-Paez et al., 2014). Transiently arresting the cells in G2-phase reduced the structural defects, which indicated that cell with lower levels of Smc5 and Smc6 required more time to complete replication.

In the projects presented in this thesis, we have discovered new functions of Smc5/6 in DNA replication, chromosome segregation and the maintenance of stalled forks caused by HU. These functions are summarized in the Results and Discussion chapter, and detailed descriptions are found in **Paper I**, **Paper II**, and **Paper IV**.

CHROMOSOMAL ASSOCIATION OF SMC COMPLEXES

The SMC complexes perform their functions through the association with chromosomes. Therefore, knowledge about when and where they associate with chromosomes can lead to better understanding of the functions of SMC complexes. Their association with chromosomes is highly regulated during the cell cycle. The complexes do not associate with specific recognition sequences. Instead chromosomal features, such as centromeres and gene orientation, are important factors in the localization of SMC complexes. The chromosomal association of cohesin and Smc5/6 are introduced in the sections below.

The chromosomal association of cohesin

Cohesin is loaded onto chromosomes before replication by the Scc2-Scc4 complex (Ciosk et al., 2000). At this stage, cohesin's association with chromosomes is dynamic, since Wapl promotes cohesin dissociation and Scc2-Scc4 continuously loads new complexes (Chan et al., 2012; Gerlich et al., 2006; Lopez-Serra et al., 2013). During replication, when sister chromatid cohesion is established, a subset of cohesin complexes become stably associated with chromosomes. This is achieved by the acetylation of Smc3 by Eco1, which counteracts Wapl's destabilizing activity against cohesin (Rolef Ben-Shahar et al., 2008; Sutani et al., 2009; Unal et al., 2008). In human cells, Smc3 acetylation leads to the recruitment of sororin, which is also required to counteract Wapl (Nishiyama et al., 2010; Schmitz et al., 2007).

Detailed chromatin immunoprecipitation (ChIP) analyses in yeast have shown that cohesin localizes at core centromeres and along chromosome arms in between convergently oriented genes (Lengronne et al., 2004; Tanaka et al., 1999). The loading complex, Scc2-Scc4, is however not found at the cohesin sites on chromosome arm. Instead it is found at core centromeres and highly transcribed genes (Hu et al., 2011; Lengronne et al., 2004). These findings have led to a model in which cohesin is loaded at Scc2-Scc4 sites, and then relocates by being pushed by transcription machineries to finally reside in between

convergently oriented genes (Lengronne et al., 2004). The relocation from the initial loading sites was later suggested to dependent on ATP hydrolysis of the complex (Hu et al., 2011).

Cohesive cohesin complexes need to be removed from chromosomes to allow chromosome segregation in anaphase. In human cells, this is completed through a two-step mechanism. First Wapl promotes dissociation of cohesin from chromosome arms, in a pathway called the prophase pathway (Kueng et al., 2006; Waizenegger et al., 2000). The remaining cohesin around centromeres is then cleaved by separase at anaphase onset (Hauf et al., 2001). In *S. cerevisiae*, a prophase pathway does not exist, instead all cohesin complexes are cleaved at anaphase onset (Uhlmann et al., 1999).

The reloading of cohesin after anaphase, starts already in telophase in human cells (Gerlich et al., 2006), whereas it occurs in late G1-phase in *S. cerevisiae* (Michaelis et al., 1997; Uhlmann and Nasmyth, 1998). This is explained by that Scc1 is not present in *S. cerevisiae* cells until late G1, since all Scc1 was cleaved in anaphase. The fact that the chromosomal binding pattern of *S. cerevisiae* cohesin in late G1 is indistinguishable from the pattern seen after DNA replication (Lopez-Serra et al., 2013), shows that no new binding sites are created during cohesion establishment. To date, it is also unknown if cohesion establishment occurs at all cohesin sites. However, a study in human cells showed that acetylated Smc3 was only present at a small subset of cohesin sites (Deardorff et al., 2012). This indicates that cohesion establishment does not occur at all cohesin sites on chromosomes during replication.

Lastly, cohesin is also enriched around an induced DNA double strand break, and establishes new cohesion throughout the genome in response to DNA damage (Strom et al., 2007; Unal et al., 2007).

The chromosomal association of Smc5/6

Using ChIP-on-chip in *S. cerevisiae*, Smc5/6 was shown to associate around centromeres and at various positions along chromosome arms. Unlike cohesin, this association occurred specifically after replication (Lindroos et al., 2006). An interesting finding in this study was that Smc5/6 displayed a chromosome-length dependent binding pattern, with a higher density of binding sites on longer chromosomes, compared to short chromosomes. Smc5/6 was also found to be enriched at or around the rDNA (Lindroos et al., 2006; Torres-Rosell et al., 2005). In addition, the complex was found to accumulate around an induced DNA double

strand break (De Piccoli et al., 2006; Lindroos et al., 2006). The accumulation at DNA breaks, unlike the association to the rest of the undamaged genome required Mre11, a member of the MRX complex that is involved in the initial processing of DNA breaks (Lindroos et al., 2006). In this study, Smc5/6 was also shown to accumulate around replication forks stalled by HU in the absence of a functional checkpoint (Lindroos et al., 2006). In human cells, the chromosomal association of Smc5/6 has been analyzed by chromatin fractionation and microscopy. These assays showed that Smc5/6 associated with chromatin in interphase, but largely dissociated in mitosis when chromosomes were condensing (Gallego-Paez et al., 2014).

Analysis of the chromosomal association of Smc5/6 has been one of the main focuses of this thesis. Our findings are summarized in the Results and Discussion chapter, and described in detail in **Paper I**, **Paper II**, **Paper III** and **Paper IV**).

METHODOLOGY

In this chapter the model organism and the principal methods used in the thesis are described.

MODEL ORGANISM

In all four papers presented in this thesis, the budding yeast *S. cerevisiae* was used as model organism. This unicellular eukaryote represents an excellent experimental system since it has a short life cycle (of around 90 minutes in ideal conditions), is easy to cultivate, and to genetically manipulate.

The *S. cerevisiae* genome was the first eukaryotic genome to be completely sequenced (Dujon, 1996; Goffeau et al., 1996). *S. cerevisiae* has 16 linear chromosomes with a total of just over 12 million bp, excluding the multiple rDNA repeats on chromosome 12 which can vary in number. The shortest chromosome, chromosome 1, has a length of 230 kb, and the longest, chromosome 12, has a length of approximately 2350 kb including the rDNA array. The *S. cerevisiae* genome contains around 6000 genes with an average ORF length of 1450 bp. This means that the genome is highly gene dense, with more than 70 % of the genome consisting of ORFs. The intergenic regions are therefore very short, for example with an average of only 326 bp in between convergently oriented ORFs. In addition, only 4 % of genes have introns (Dujon, 1996).

This small and gene dense genome, consisting of many relatively short chromosomes, has obvious distinctions from genomes of many multicellular eukaryotes, which are larger and more complex. However, research using *S. cerevisiae*, in which many processes and proteins are conserved to human cells, has proven to be an excellent approach by which a less complicated system is used to ask complex questions and discover basic mechanism, which later can be addressed in human cells. One example related to this thesis, is the discovery of how sister chromatids are held together by the cohesin complex, which was first discovered in *S. cerevisiae* and later proven to be well-conserved in human cells. In addition, working in a system that allows great detail and highly controlled experiments has strong potential to lead to unexpected findings. One examples of this is found in **Paper I**, where we could analyze the replication timing of specific chromosomes, which is considerably more difficult in multicellular eukaryotes.

CHROMATIN IMMUNOPRECIPITATION

ChIP is a technique that allows the analysis of where a specific target protein associates to chromosomes. This type of information adds important details to the understanding of chromosome-related functions of target proteins. The first step of the ChIP method is to grow cells under desired conditions. Cells are then harvested and treated with formaldehyde, which crosslinks proteins bound to chromosomes. After cell lysis, chromatin is sheared by sonication into fragments of around 300-500 bp in length. Using a specific antibody, the target protein is then immunoprecipitated, which also brings down the DNA fragments that are crosslinked to the protein. Thereafter, crosslinks are reversed and DNA is purified for downstream analysis (Figure 5) (Katou et al., 2006).

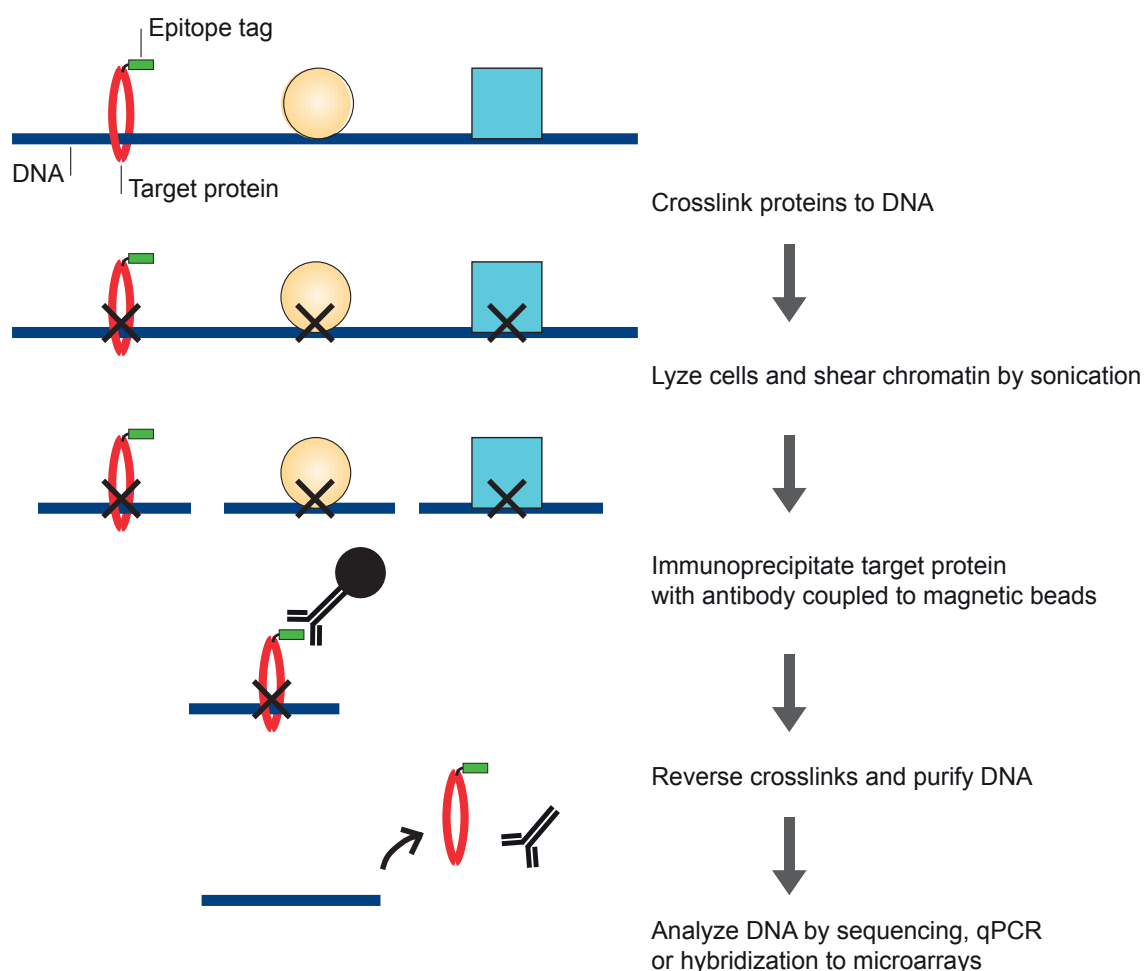


Figure 5. Chromatin immunoprecipitation workflow

Overview of chromatin immunoprecipitation. In this schematic, the target protein is labeled with an epitope tag (green rectangle), which is recognized by antibodies coupled to magnetic beads.

The amount of DNA for specific loci in the ChIP fraction relative to the amount in the input fraction is then analyzed using tiling microarrays (ChIP-on-chip), massive parallel sequencing (ChIP-seq) or quantitative PCR (ChIP-qPCR). Both ChIP-on-chip and ChIP-seq allow genome-wide analysis of the chromosomal association of a protein of interest in a single experiment, however ChIP-seq provides higher spatial resolution and signal-to-noise ratio than ChIP-on-chip (Ho et al., 2011). ChIP-qPCR on the other hand provides fully quantitative data for specific loci.

ChIP is a population-based assay and the obtained results represent an average of a target proteins binding profile in the population. Therefore it is not possible from a single experiment to know if weak signal at a particular locus, relative to another locus, is due to that fewer cells in the population have the target protein bound to that site, or if the target protein has a more dynamic association to that specific site. To exclude false positive binding sites, it is important to use the proper controls. For ChIP-on-chip and ChIP-seq, one way to do so is to analyze the input fraction, and not only the ChIP fraction (Ho et al., 2011; Nakato et al., 2013). In *S. cerevisiae*, experiments are often performed using epitope-tagged target proteins, and an antibody recognizing the epitope tag. This allows for a more detailed control to exclude false positive binding sites, by performing experiments on cells lacking the epitope tag on the protein of interest. In addition, the same epitope tag and high quality antibody can be used to study any target protein of choice, which minimizes experimental differences between studies of different proteins. Although there are advantages of using epitope tagged target proteins, it is important to always control that the small epitope tag does not interfere with the target protein's function.

TWO-DIMENSIONAL GEL ELECTROPHORESIS

Two-dimensional gel electrophoresis analysis is a powerful technique that visualizes DNA structures present at specific sites in the genome. This allows for analysis of replication fork progression through, or homologous recombination within, any chromosomal locus of interest. The technique is based on the finding that branched and linear DNA molecules of the same molecular mass can be separated by gel electrophoresis (Bell and Byers, 1983). Briefly, genomic DNA is carefully purified and then digested using restriction enzymes. The restriction enzymes should be chosen so that the locus of interest resides close to the center of a 3-6 kb restriction fragment. The digested DNA is separated by gel electrophoresis in the first dimension, using low agarose concentration and voltage. This results in a separation

largely based on the mass of the DNA molecules. The sample lanes are then excised and the second dimension gel electrophoresis is performed in a 90 degrees angle to the direction of the first dimension. In the second dimension the higher voltage and agarose concentration, and the fact that the intercalating agent ethidium bromide is added, contribute to that the separation of DNA molecules is not only based on mass but also on their structure. Using the standard Southern blot technique, the DNA is then transferred to a membrane and detection of the locus of interest is achieved using a specific radiolabeled probe. The results show the characteristic pattern of replication or recombination intermediates present within the locus of interest (Figure 6) (Friedman and Brewer, 1995).

The technique was first developed to detect replication origins on plasmids (Brewer and Fangman, 1987), but has been extended to monitor replication progression on linear chromosomes (developed in (Brewer and Fangman, 1988) and used in **Paper II** and **Paper III**), replication termination (Fachinetti et al., 2010), recombination intermediates at stalled replication forks (developed in (Branzei et al., 2006) and used in **Paper IV**), hemicatenane

formation (developed in (Lopes et al., 2003) and used in **Paper II**). Related to **Paper II** and **Paper III**, SCIs are fully replicated sister chromatids that are wrapped around each other. Therefore, restriction digestion of the genome into smaller fragments will dissolve them, which prevents the use of the two-dimensional gel electrophoresis technique to detect SCIs. Hemicatenanes or recombination intermediates on the other hand, have more stable junctions between the sister chromatids that allows their detection.

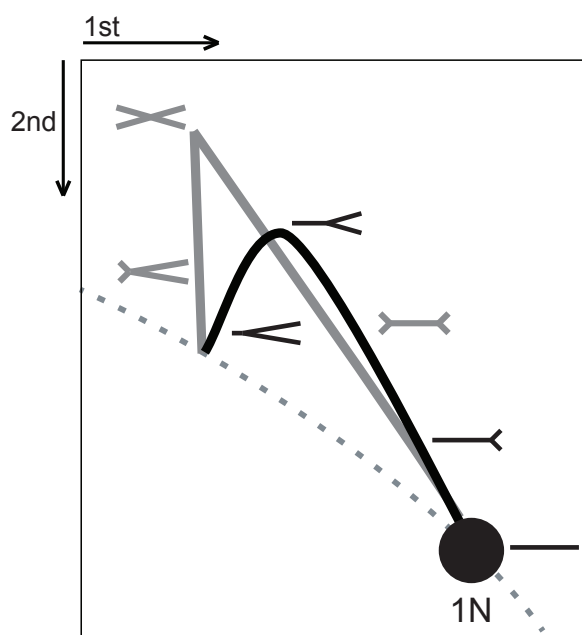


Figure 6. Replication intermediates visualized by two-dimensional gel electrophoresis

Schematic representation of replication intermediates detected by two-dimensional gel electrophoresis. The 1N spot represents linear DNA molecules, in which a replication fork is not present in the investigated fragment.

ADDITIONAL TECHNIQUES IMPORTANT FOR THE THESIS

In **Paper I**, replication completion of specific chromosomes was monitored by pulse-field gel electrophoresis (PFGE). Since *S. cerevisiae* chromosomes are relatively short, they can penetrate and be separated on agarose gels in their intact form, as long as they are purified from non-replicating cells. However, the branched structures of replicating chromosomes prevent entry into the gel (Hennessy et al., 1991). Therefore, only chromosomes that have completed, or not commenced, replication can be resolved by PFGE. To prevent the visualization of chromosomes that have not commenced replication, cells were made to replicate in the presence of bromodeoxyuridine (BrdU) during a single S-phase. When bulk replication appeared complete by the standard fluorescence-activated cell sorting analysis (FACS) method, cells were harvested and chromosomes were separated by PFGE. The chromosomes were then transferred to a membrane and chromosomes were detected using an anti-BrdU antibody. The signal for each specific chromosome was then quantified relative to the total signal of all chromosomes, including those in the well that did not penetrate the gel. This assay allows the quantitative detection of the timing of replication completion of specific chromosomes.

In **Paper I** and **Paper II**, plasmid assays were used to monitor SCIs dynamics. Since SCIs cannot be directly visualized on linear chromosomes, direct analysis of SCIs has to be performed on circular plasmids, which remain intertwined during DNA preparation as long as Top2 is kept inactive. In the plasmid assays used for the two papers in this thesis, reporter plasmids were introduced into *S. cerevisiae* cells and their topology was analyzed using standard gel electrophoresis and Southern blotting.

In **Paper II**, live-cell imaging was used to monitor chromosome segregation. As part of this technique, individual cells are followed through mitosis, carrying a fluorescently labeled chromosomal locus. The technique allows detailed studies of when and at what positions sister chromatids separate from each other, and when the two chromatids successfully segregate into the mother and daughter cells. To visualize chromosomes, tetracycline operators were integrated at a specific locus. The cells also express tetracycline repressors, tagged with the fluorescent protein td-Tomato, which bind to the operators with high specificity. Tubulin was also tagged with another fluorescent protein, GFP, which allowed spindle elongation to be monitored. By measuring spindle length using a computer software, the definition of a specific time point in anaphase could be established, *i.e.* when the spindle had reached a defined length. The results from this assay were presented as when the sister chromatids separated from each other (at the locus of interest) relative to spindle elongation,

and when the sister chromatids (again at the locus of interest) segregated successfully into the mother and daughter cell relative to the time of their separation.

RESULTS AND DISCUSSION

The four papers presented in this thesis are centered on Smc5/6, its association to chromosomes and its functions during DNA replication and chromosome segregation. The findings of each paper are presented and discussed below.

PAPER I

This investigation was started based on the previous intriguing finding that Smc5/6 associates to chromosomes in a chromosome-length dependent manner, with more binding sites per kb on longer chromosomes as compared to shorter ones (Lindroos et al., 2006). Since Smc5/6 did not associate with specific recognition sequences or established chromosomal features, we hypothesized that the level of topological stress on chromosomes was an important factor for Smc5/6 chromosomal recruitment. Transcription-induced topological stress has been suggested to dissipate over chromosome ends in *S. cerevisiae* (Joshi et al., 2010). Longer chromosomes might therefore suffer from increased topological stress, as the distance to an “open” chromosome end is longer for a larger part of the chromosome. Supporting our hypothesis, the absence of Top1 or Top3 specifically delayed the replication of long *S. cerevisiae* chromosomes. A similar phenotype was found for Smc5/6 mutants, which created the possibility that the complex helps to reduce topological stress during replication of long budding yeast chromosomes.

Using ChIP-on-chip, Smc5/6 association with chromosomes was then shown to require DNA replication. Since Smc5/6 associates with higher density to longer chromosomes, this could also be a reflection of the topological status of the chromosome during S-phase. To further investigate this, Smc5/6 chromosomal association was assayed in *top2* mutants and in cells treated with camptothecin (CPT) to inactivate Top1. The presence of CPT did not alter Smc5/6 chromosomal association. However, after an S-phase in the absence of Top2 function, the number of Smc5/6 chromosomal binding sites was significantly increased. Inactivation of Top2 still allows the completion of replication (Bermejo et al., 2007), but increases the number of SCIs, as judged by chromosome breakage during segregation (Spell and Holm, 1994) and plasmid assays (DiNardo et al., 1984; Koshland and Hartwell, 1987). Therefore, Smc5/6 chromosomal association could be triggered by the presence of SCIs. The increase in Smc5/6 chromosomal binding sites in *top2* mutant cells was strongest on chromosomes of intermediate length. We speculated that this reflected the potential for SCIs to move along chromosomes and resolve passively over chromosome ends. On long

chromosomes, SCIs might be stable even in wild-type cells, due to low level of passive resolution over chromosome ends. However on chromosomes of intermediate length, SCIs might be less stable in wild-type cells and an increased number of SCIs could be more easily detected. On the shortest chromosomes, any potential increase of SCIs due to the absence of Top2 during replication might be reduced since SCIs more easily can dissipate over chromosome ends.

To further challenge the idea that Smc5/6 chromosomal association was triggered by SCIs, ChIP-on-chip of Smc5/6 was performed in cells carrying either the short chromosome 3 in a circularized form, or the long chromosome 4 divided into two fragments. The results showed that Smc5/6 is strongly enriched on the circular version of chromosome 3, as compared to the linear version. This result could indicate that in the absence of chromosome ends, SCIs are stabilized and can no longer be passively resolved by rotation of “open” chromosome ends. This is supported by the observation that short circular chromosomes break, unlike linear ones, during chromosome segregation in the absence of Top2 function (Spell and Holm, 1994). When the long chromosome 4 is present as two fragments, each with their own centromere, Smc5/6 peak density is reduced, as compared to on the endogenous chromosome 4. The lower levels of peak density on the new shorter chromosomes are similar to those on natural chromosomes of the same length. These results further strengthen the finding that Smc5/6 association to chromosomes is more influenced by chromosome length than sequence or any other established chromosomal feature.

Smc5/6 was then shown to promote the intertwining of a reporter plasmid in the absence of Top2 function. The experiments were performed in the absence of Top2 function, since at that time intertwined plasmids were not detectable in wild-type cells due to technical limitations. In *top2 smc6* double mutant, less intertwined dimers and more supercoiled monomeric form of the reporter plasmids were found, as compared to a *top2* single mutant. This result suggested that Smc5/6 promotes the formation of intertwinings by assisting replication fork rotation. Altogether the results allowed us to propose a speculative model in which Smc5/6 promotes fork rotation by sequestering SCIs behind the fork. This would reduce positive supercoils ahead of the replication fork to allow replication of regions with high topological stress.

The finding that Smc5/6 promote replication fork rotation and therefore the intertwining of plasmids was later challenged by Farcas and colleagues (Farcas et al., 2011). They developed a technique that for the first time allowed the detection of intertwined

plasmids in wild-type cells. Their results show that Smc6 function had no obvious effect on the formation of intertwined plasmids. Several possibilities can account for the differences in the two studies. We used small plasmid (4.6 kb) and detected how Smc5/6 affected its topological status in the absence of Top2 function, while Farcas and colleagues used a 26 kb plasmid to investigate the role of Smc5/6 in wild-type background. In addition, the temperature-sensitive alleles used to inactivate Smc5/6 were different in the two studies. Additional experiments are required to understand the difference in results by the two studies.

PAPER II

This investigation was started to challenge the hypothesis that Smc5/6 chromosomal association is triggered by SCIs, as presented in **Paper I**. After the publication of **Paper I**, cohesin was shown to protect SCIs from resolution by Top2 on long plasmids (Farcas et al., 2011). If Smc5/6 chromosomal association is indeed triggered by SCIs, it would be expected to be dependent on cohesin function. However, a previous investigation showed, using ChIP-on-chip, that Smc5/6 chromosomal binding pattern in a cohesin mutant was altered into more numerous, jagged and narrow peaks, which indicated that cohesin controlled the positioning of Smc5/6 on, but not the association to, chromosomes (Lindroos et al., 2006). If, as this result suggested, Smc5/6 remained associated with chromosomes although the sister chromatids prematurely separated due to nonfunctional cohesin, it would be difficult to reconcile with an SCI-dependent association.

To explore this in more detail, we first decided to revisit cohesin's role in Smc5/6 chromosomal association using ChIP-seq and ChIP-qPCR, which provide higher resolution and quantitative data, respectively. Our new results clearly showed that Smc5/6 required the function of cohesin and the cohesin loader Scc2 to associate with chromosomes. The reason why the previous study (Lindroos et al., 2006) failed to detect that Smc5/6 chromosomal association is dependent on cohesin remains unknown. However, it does not depend on the lower resolution of the ChIP-on-chip assay compared to ChIP-seq. This is because the previous study could detect that Smc5/6 chromosomal association is dependent on the Scc2, and our new quantitative results showed that the absence of cohesin or its loader reduce Smc5/6 chromosomal association to the same low levels. In addition, the similarly low levels of Smc5/6 in Scc2 and cohesin mutants also suggest that the role of Scc2 in the chromosomal association is mediated through cohesin loading and not through direct loading of Smc5/6.

Our finding that cohesin and its loader Scc2 are required for Smc5/6 chromosomal association was strengthened by experiments using Eco1 and Pds5 mutants. Both mutants fail to establish sister chromatid cohesion (Chan et al., 2013; Skibbens et al., 1999; Toth et al., 1999). In the absence of Eco1 function, cohesin is loaded onto chromosomes but cohesion is not established (Skibbens et al., 1999; Toth et al., 1999). The fact that Smc5/6 did not associate with chromosomes in this mutant shows that cohesin loading onto chromosomes is not sufficient to promote Smc5/6 association. Instead the chromosomal association of Smc5/6 also requires cohesion establishment. In the absence of the cohesion antiestablishment factor Wapl (Wpl1 in *S. cerevisiae*), Eco1 becomes largely dispensable for cohesion establishment, and the *eco1 wpl1* double mutant is viable (Rolef Ben-Shahar et al., 2008; Sutani et al., 2009; Unal et al., 2008). In this double mutant Smc5/6 also associated with chromosomes at wild-type levels. Altogether these results show that chromatids need to be held together for Smc5/6 to associate to the replicated genome.

The use of ChIP-seq provided a higher resolution of the specific binding sites, and the use of no tag-control experiments allowed us to exclude several false positive binding sites from the previous investigation. In wild-type background, strong Smc5/6 binding sites were found at core centromeres and in between centromere-proximal convergently oriented genes. At these sites Smc5/6 co-localized with cohesin. Cell-cycle experiments also showed that Smc5/6 enrichment at these sites was low or absent in G1-phase, reached its peak in G2/M-phase, and disappeared in anaphase. By focusing on the Smc5/6 enrichment in pericentromeric regions, where strong signals were detected, and correlating it to chromosome length we found a clear positive correlation (Figure 7A). In agreement with previous investigations, (Lindroos et al., 2006) and **Paper I**, this showed that more Smc5/6 was found in pericentromeric regions on longer chromosomes as compared to short ones. Since *S. cerevisiae* chromosome ends had been suggested to be topologically “open” (Joshi et al., 2010), we decided to see if the proximity to a chromosome end correlated with the Smc5/6 enrichment in pericentromeric regions. Interestingly, the correlation to the length of the shorter chromosome arm was even stronger than the correlation to whole chromosome length (Figure 7B). These two different correlation analyses are in part overlapping, since longer *S. cerevisiae* chromosomes more often have longer shorter arms, than shorter chromosomes. Nevertheless, especially for the longer chromosomes the correlation was improved. Our previous interpretation on the length-dependency of Smc5/6 chromosomal association, presented in **Paper I** (Figure 7C), was based on that SCIs would be able to swivel off chromosome ends more easily on shorter chromosomes as compared to long ones.

Based on these new findings, we instead speculated that the topological stress during replication in the pericentromeric region is dependent on the distance to an “open” chromosome end. Pericentromeric regions found further away from a telomere might therefore suffer from higher levels of topological stress, which causes more replication fork rotation and thereby leaves more SCIs behind, bound by Smc5/6.

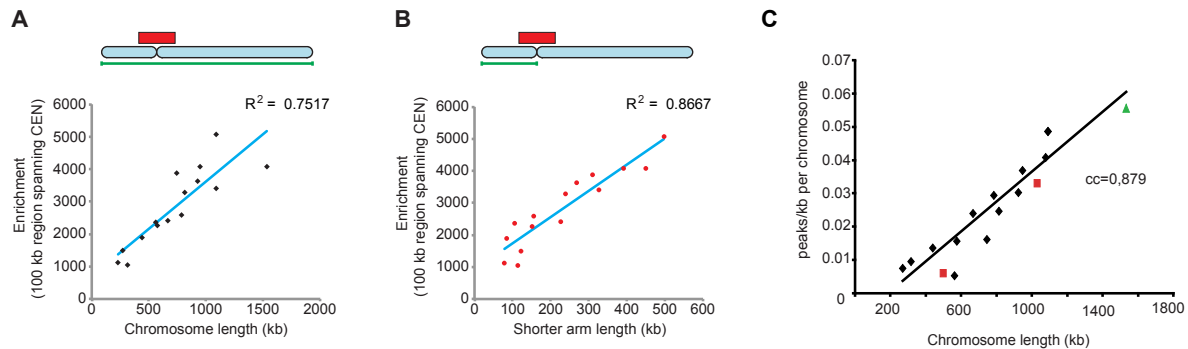


Figure 7. Chromosome length, and the length of the shorter chromosome arm, correlates with Smc5/6 chromosomal enrichment around centromeres.

(A) Smc5/6 enrichment in pericentromeric regions, as determined by ChIP-seq, correlates with chromosome length (**Paper II**). (B) The correlation of Smc5/6 enrichment in pericentromeric regions with the length of the shorter chromosome arm is enhanced, as compared to whole chromosome length (**Paper II**). (C) The peak density (binding sites per kb) of Smc5/6, as determined by ChIP-on-chip, correlates with chromosome length (**Paper I**).

In **Paper I**, we found that Smc5/6 accumulates on chromosomes in a *top2* mutant. In this study using ChIP-seq and ChIP-qPCR, we confirmed this finding and could add new details due to the higher resolution and more quantitative data obtained by these assays. First, the accumulation of Smc5/6 in *top2* mutant cells occurred only on chromosome arms and not around centromeres. Secondly, the new Smc5/6 binding sites were found in between convergently oriented genes and co-localizing with cohesin. The chromosomal association pattern of cohesin was shown not to be affected by Top2 inactivation, ruling out the possibility that the Smc5/6 accumulation was caused by a change in cohesin binding pattern. The Smc5/6 chromosomal association in *top2* mutant cells was shown to be dependent on cohesin by ChIP analyzes, although some remaining chromatin association was detected using chromosome spread analysis. Inactivation of Top2 during a G1- or G2/M-arrest did not change the chromosomal association pattern of Smc5/6. These findings show that Top2 needs to be inactive during replication to cause accumulation of Smc5/6 on chromosome arms. This also suggests that it is not the absence of Top2’s function in transcription that results in additional Smc5/6 chromosome binding.

Smc5/6 had previously been shown to accumulate at DNA double strand breaks and to function in homologous recombination (De Piccoli et al., 2006; Lindroos et al., 2006; Torres-Rosell et al., 2005), so the possibility existed that DNA breaks occurred at cohesin sites in *top2* mutant cells, which then triggered the recruitment of Smc5/6. However, we found that the DNA damage checkpoint, as indicated by Rad53 phosphorylation, was not activated after cells had replicated in the absence of Top2 function. In addition, in the absence of Mre11, a factor required for Smc5/6 to accumulate at DNA breaks (Lindroos et al., 2006), Smc5/6 accumulated on chromosome arms when Top2 function was inactivated. In the absence of Rad52, cells cannot perform canonical homologous recombination (Krogh and Symington, 2004; Paques and Haber, 1999). Since Smc5/6 accumulated on chromosome arms when Top2 function was impaired also in *rad52Δ* mutant background, this shows that it is not Rad52-mediated homologous recombination at cohesin sites in *top2* mutant cells that recruits Smc5/6. Taken together, these results indicate that Smc5/6 chromosomal binding in the absence of Top2 function is not caused by DNA double strand breaks or recombination intermediates.

Top2 had previously been shown to promote replication termination (Fachinetti et al., 2010). In *top2* mutant cells, termination was slightly delayed as compared to wild-type cells. This created the possibility that Smc5/6 association in *top2* mutant cells in G2/M-arrest, was due to incomplete replication, and that Smc5/6 associated to the remaining replication forks. However, ChIP-seq of a polymerase epsilon subunit after an S-phase in a *top2* mutant did not reveal any enrichment of the polymerase on chromosomes. Furthermore, the Smc5/6 chromosomal binding pattern was not altered in the absence of the Rrm3 helicase, as detected by ChIP-on-chip. In Rrm3 mutant cells, replication fork pausing occurs at specific sites in the genome (Ivessa et al., 2003; Ivessa et al., 2000). Our logic for this experiment was that if Smc5/6 binding was due to fork pausing, we expected to see more Smc5/6 on chromosomes in the absence of Rrm3. This finding was later challenged by quantitative ChIP-qPCR analysis in **Paper III** and is discussed in detail below. More importantly, however, Smc5/6 sites were investigated by two-dimensional gel electrophoresis, which showed that Smc5/6 chromosomal association in *top2* mutant cells is not due to the persistence of replication forks in G2/M-arrest.

We then decided to analyze if restored Top2 activity after replication was able to remove Smc5/6 that had accumulated on chromosomes due to the absence of Top2 during replication. It had previously been showed that Top2 is required only at the time of mitosis to ensure cell survival (Holm et al., 1985). This suggests that Top2 can resolve any accumulated

entanglements if it is reactivated just before, or during, chromosome segregation. We confirmed this using live-cell imaging to study chromosome segregation. In addition, using a small reporter plasmid, we showed that Top2 reactivation led to resolution of an intertwined plasmid during a G2/M-arrest. After these control experiments, Smc5/6 was shown, using ChIP-seq and ChIP-qPCR, to dissociate from chromosomes when Top2 activity was restored in G2/M-arrest. Smc5/6 enrichment was reduced to levels seen in wild-type background, showing that the excess of Smc5/6 on chromosomes was removed. If Top2 was kept inactivated during a prolonged G2/M-arrest, there was no reduction in Smc5/6 enrichment levels, further strengthening the conclusion that the accumulation of Smc5/6 in *top2* mutant cells is not due to a delay in replication termination. These results indicate that Smc5/6 associates to a chromosomal structure that Top2 can resolve after DNA replication.

Since Top2 inactivation after S-phase did not affect Smc5/6 chromosomal association, this created the possibility to analyze segregation of chromosomes in the absence of Top2 function during mitosis with high or low levels of Smc5/6 binding, resulting from the inactivation of Top2 before or after replication, respectively. Chromosome segregation was again investigated by live-cell imaging, and the results showed that chromosomes with higher levels of Smc5/6 enrichment missegregated and had more difficulty in separating its sister chromatids, than the same chromosomes with lower level of Smc5/6 enrichment. These results show that Smc5/6 enrichment on chromosomes correlate with the segregation-inhibiting structures present on chromosomes in *top2* mutants. These structures are most likely SCIs, but the development of assays capable of directly visualizing these structures is needed to confirm this hypothesis.

Lastly, we posed the question if Smc5/6 had a function in the segregation of entangled chromosomes. Thus, we decided to analyze chromosome segregation in the absence of both Top2 and Smc5/6 functions. The experiment was performed by investigating the shortest *S. cerevisiae* chromosome, since this chromosome is able to segregate correctly in the majority of *top2* mutant cells, likely due to the passive resolution of SCIs over chromosome ends. Longer chromosomes missegregate at high rates in *top2* mutant cells, which makes it difficult to address if Smc5/6 also has a segregation-promoting function on these chromosomes. The results showed that the shortest chromosome missegregates three-fold as often in *top2 smc6* double mutants, as compared to *top2* single mutants. The increased missegregation was not due to a failure in the removal of cohesin, which was proposed to occur in *S. pombe top2 smc6* double mutants (Outwin et al., 2009). The authors of that study suggested that *top2 smc6* mutants failed to remove a subset of cohesin complexes, which are normally removed

by a separase-independent pathway in *S. pombe*. In *S. cerevisiae* this pathway does not exist (Schmidt et al., 2009), instead all cohesin complexes are cleaved during anaphase (Ciosk et al., 2000; Uhlmann et al., 1999). Our results show that Smc5/6 either promotes Top2-independent resolution of SCIs, or prevents aberrant linkages on short entangled chromosomes that cannot be passively resolved. Future investigations will be needed to analyze if these aberrant sister chromatid linkages require recombination to be formed. It would also be interesting to see if restored Top2 activity can resolve them, after an S-phase in the absence of both Top2 and Smc5/6 functions.

Altogether, our results suggest that Smc5/6 indicates the positions where chromosomes are entangled. If so, the results are in favor of that cohesin directly protects SCIs also on linear chromosomes, at least in pericentromeric regions. Smc5/6 is also shown to promote the segregation of entangled sister chromatids. Furthermore, the results strengthen the possibility that topological stress during S-phase is important for the formation of SCIs.

PAPER III

This investigation was started to further explore the hypothesis, presented in **Paper II**, that topological stress during S-phase is one of the factors that determines if Smc5/6 associates to chromosomes, potentially by promoting SCI formation. Topological stress in the form of positive supercoils accumulates ahead of translocating enzymatic machineries, such as the replisome and RNA polymerases, when they unwind the DNA helix (Brill et al., 1987; Liu and Wang, 1987). Consistently, strong convergent transcription results in the accumulation of high levels of topological stress (Garcia-Rubio and Aguilera, 2012). To locally increase the topological stress in the *S. cerevisiae* genome, we therefore artificially increased the transcription levels of two closely situated convergently oriented genes, *MCRI* and *DBRI*. Thereafter, ChIP-on-chip and ChIP-qPCR were used to assay the chromosomal association of Smc5/6. The increase in transcription levels was achieved by replacing the endogenous promoters of the two genes with strong constitutively active promoters. The results showed that Smc6 specifically accumulated to a high level in between the two genes when they were overexpressed.

In **Paper II**, we found that Smc5/6 co-localizes with cohesin in both wild-type and *top2* mutant cells. In addition, the enrichment levels of the two complexes at individual binding sites correlated. Cohesin was previously suggested to be pushed by the transcription machinery to finally reside in between convergently oriented genes (Lengronne et al., 2004).

Therefore, the overexpression of the two convergently oriented genes, *MCRI* and *DBRI*, could lead to the positioning of more cohesin, which in turn could allow more Smc5/6 binding. However, this possibility was excluded since ChIP-qPCR of Scc1 (a cohesin subunit) showed that there was no increase in cohesin enrichment by high *MCRI* and *DBRI* transcription.

We then used two-dimensional gel electrophoresis to monitor replication fork progression through the two genes, and found that the increased transcription also resulted in distinct replication fork pausing. The pausing signal was seen strongest in mid-S-phase, and was almost completely absent when bulk replication was finished. The fork pausing was largely dependent on the increased transcription of the *DBRI* gene, which orientation opposes the incoming fork. Overexpression of *MCRI* alone, which is oriented co-directionally with replication fork progression, did not cause detectable fork pausing. Hence, the pausing is due to head-on-collision of the replication fork and the transcription machinery. Our data is in agreement with the previous finding that highly expressed RNAPII genes on plasmids pause the replication fork only when they are oriented against the incoming replication fork (Prado and Aguilera, 2005). This is also consistent with that fork pausing seen at RNAPIII-transcribed genes is limited to head-on-collisions (Deshpande and Newlon, 1996). However, a ChIP-on-chip-based study by Azvolinsky and colleagues suggested that highly expressed RNAPII-genes pause the replication fork independently of their orientation (Azvolinsky et al., 2009). The experimental rationale of this study was to detect replication fork pausing by ChIP-on-chip of the DNA polymerase epsilon subunit, Pol2, in asynchronously growing cells. Potentially, the ChIP-on-chip-based study suffers from detecting false-positive “pausing sites”, since a later study revealed that ChIP has the potential to detect erroneous signals within highly expressed ORFs (Teytelman et al., 2013). This is further supported by that the authors were unable to confirm distinct fork pausing in genes oriented co-directionally with the replication fork using two-dimensional gel electrophoresis. The finding that high transcription can produce artifactual signals within ORFs highlights the importance of validating ChIP-on-chip data by the use of ChIP-qPCR and “no tag”-controls, when possible. In addition, to further ascertain that the investigated ChIP-signal at a highly transcribed locus is not due to a false positive signal, it can be useful to, if possible, separate the high transcription from the accumulation of the target protein. This can be achieved by, for example, looking at different cell cycle stages or in mutants in which one of the events (binding of the target protein or transcription levels) is altered, but not the other.

In our study, high convergent transcription resulted in both local Smc5/6 chromosomal association and replication fork pausing. Therefore, the possibility existed that the transcription-induced Smc5/6 enrichment in between the two genes was due to direct association with the paused fork. However, more detailed analysis of the location of fork pausing showed that it occurs within the *DBR1* ORF, whereas Smc5/6 enrichment peaks in between the two overexpressed genes. This spatial discrepancy argued against that Smc5/6 directly associated to the paused fork.

To further explore the correlation between the two events, we sought for factors regulating fork pausing with the idea to test if they also affected Smc5/6 enrichment. We found that in the absence of the helicase Rrm3, fork pausing was strongly enhanced in mid-S-phase. However, in the end of S-phase no paused replication forks were detected, similar to wild-type background, indicating that the forks had resumed, or replication termination had occurred at the paused fork. Rrm3 moves with the replication fork (Azvolinsky et al., 2006), and assists it past obstacles consisting of non-histone proteins bound tightly to DNA, for example at centromeres, inactive origins, RNAPIII-genes and in the rDNA (Ivessa et al., 2003; Ivessa et al., 2000). However, Rrm3's role in assisting replication forks past highly expressed RNAPII-transcribed genes is controversial. The plasmid-based study mentioned above found that Rrm3 counteracts fork pausing when a replication fork meets an opposing highly expressed RNAPII gene (Prado and Aguilera, 2005), whereas the ChIP-on-chip-based study, also mentioned above, suggested that Rrm3 had no role in assisting the fork past highly expressed RNAPII genes in the unaltered genome (Azvolinsky et al., 2009). Our data is in agreement with the plasmid-based study. The finding of Azvolinsky and colleagues that Rrm3 has no role in assisting the fork past highly expressed RNAPII genes was based in part on ChIP-on-chip experiments of Pol2 in asynchronously growing cells of wild-type and *rrm3Δ* background (Azvolinsky et al., 2009). As discussed above, false positive signals within highly expressed genes could have complicated such experimental approach. They did however detect distinct fork pausing at two loci using two-dimensional gel electrophoresis, at which the absence of Rrm3 did not affect the pausing signal. The difference in the results from our and Azvolinsky and colleagues's studies could be explained that we used cells passing through S-phase synchronously and took samples at two time points, whereas Azvolinsky and colleagues only used asynchronously growing cells. Our results show that the enhanced pausing seen in *rrm3Δ* background is transient and that the forks eventually pass the obstacle or replication termination occurs at the pausing site. Hence, it could be difficult to detect Rrm3's role in assisting the replication fork past highly expressed RNAPII genes

using asynchronously growing cells. Alternatively, the dissimilarity could be explained by the difference between the two endogenous loci investigated by two-dimensional gel electrophoresis by Azvolinsky and colleagues, and our artificial experimental setup. The two loci displaying distinct fork pausing in the study of Azvolinsky and colleagues contained a single highly expressed RNAPII gene opposing the replication fork (Azvolinsky et al., 2009), whereas in our study Rrm3's function was only assessed at a locus with two highly expressed convergently oriented RNAPII genes. Based on these experimental differences, future time course experiments on synchronized cells at highly expressed RNAPII genes in the unaltered genome, and at our *MCRI-DBRI* locus when only *DBRI* is overexpressed, have the potential to clarify the role Rrm3 role in assisting replication forks past these obstacles.

Having found that the absence of Rrm3 strongly enhanced fork pausing in our experimental setup, we turned to see if Rrm3 also affected Smc5/6 enrichment. ChIP-qPCR was performed in a time course experiment in wild-type and *rrm3Δ* background. In wild-type background Smc5/6 enrichment levels were low in G1-phase, increased during S-phase, peaked after bulk replication was completed, and remained high in a prolonged G2/M-arrest. This showed that Smc5/6 enrichment peaks after the fork pausing is strongest, and that Smc5/6 remains bound to the *MCRI-DBRI* locus when replication intermediates are barely detectable. Similarly, Smc5/6 enrichment levels were low in G1-phase and increased during S-phase in *rrm3Δ* background. However, when bulk replication was completed there was a drastic increase in Smc5/6 enrichment as compared to wild-type background. This increase was transient and the enrichment levels of Smc5/6 returned to the levels seen in wild-type background after extending the G2/M-arrest. The transient increase of Smc5/6 enrichment at the altered *MCRI-DBRI* locus in the absence of Rrm3 is also seen at the Smc5/6 binding sites in the unaltered genome. Since Rrm3 only assists replication fork progression at distinct sites and not all throughout the genome (Azvolinsky et al., 2006), this may indicate that all Smc5/6 binding sites are Rrm3-regulated sites. This interesting possibility warrants closer investigation in the future.

The results show that Smc5/6 enrichment after *MCRI-DBRI* overexpression is not due to the direct association to the paused fork. In **Paper II**, we proposed that Smc5/6 associates with SCIs in the unaltered genome. This might be the case also at the *MCRI-DBRI* locus. Potentially, high topological stress in this region results in fork rotation, which leaves the newly formed sister chromatids intertwined and bound by Smc5/6 in its wake, before the replication fork comes to a complete pause. However, if the Smc5/6 accumulation would represent SCIs, this would be surprising since Top2 was shown in **Paper II**, to counteract

Smc5/6 accumulation on chromosome arms. An important future experiment to challenge this idea would be to test if transcription-induced Smc5/6 accumulation requires sister chromatids to be held together by cohesin. This also has the potential to separate the pausing event from Smc5/6 enrichment, since fork pausing was not affected by the absence of cohesin function.

The transient increase in Smc5/6 enrichment seen in *rrm3Δ* background could be connected to break repair by homologous recombination, since broken forks are detected by two-dimensional gel electrophoresis. This can be addressed by testing if factors required for Smc5/6 accumulation at DNA double strand breaks, such as Mre11 (Lindroos et al., 2006), are needed for the transient increase of Smc5/6 enrichment in *rrm3Δ* background.

In summary, our data show that Smc5/6 accumulates in between *MCR1* and *DBR1* when they are overexpressed. The increased transcription also results in replication fork pausing, due to head-on-collision of the fork and the RNAPII transcription machinery. However, the Smc5/6 enrichment and fork pausing are temporally and spatially distinct. Whereas Smc5/6 enrichment peaks in late S-phase and remains high in a prolonged G2/M-arrest, the fork pausing is strongest in mid-S-phase. Smc5/6 also accumulates in between the two genes and the fork pauses within the DBR1 ORF. Enhanced fork pausing, due to the absence of the Rrm3 helicase, leads to transiently increased Smc5/6 levels, but again Smc5/6 enrichment peaks after bulk replication completion. This indicates a complex relationship between replication fork pausing and Smc5/6 enrichment. To further correlate Smc5/6 enrichment and replication fork pausing, it could be of interest to see if Smc5/6 enrichment occurs when only *DBR1* is overexpressed. Potentially, strong Smc5/6 enrichment requires high convergent transcription, unlike distinct fork pausing that only requires high transcription opposing the replication fork. Regardless, our results strengthen the hypothesis that the levels of topological stress and/or replication obstacles are important determinants in Smc5/6 chromosomal association after S-phase. In addition, our data supports the view that opposing, but not co-directional, highly expressed RNAPII transcription pauses the replication fork, and that Rrm3 assists the fork past highly expressed RNAPII-transcribed genes.

In the future, it will be important to assess the functional importance of transcription-induced Smc5/6 accumulation. One alternative could be to test if creating additional Smc5/6 binding sites on a short chromosome by the use of high convergent transcription results in that the short chromosome now needs Smc5/6 for its proper replication, as shown for longer

chromosomes with more Smc5/6 binding sites in **Paper I**. However, we did not detect any replication defects at *MCRI-DBR1* by two-dimensional gel electrophoresis in *smc6-56* mutant cells, which argues against this possibility. Instead analyzing chromosome segregation distal to the site of transcription-induced Smc5/6 accumulation in *top2-4* could be used to see if the Smc5/6 enrichment represents a structure that inhibits chromosome segregation in the absence of Top2 function. Segregation experiments could also be performed in *top2-4 smc6-56* double mutant to see if Smc5/6 function is important to segregate this locus, similar to chromosomes with many Smc5/6 sites as shown in **Paper II**.

PAPER IV

This study was started to analyze the function of the Nse5 subunit of Smc5/6. It was a collaborative study led by Jennifer Cobb's laboratory, to which Dana Branzei's laboratory contributed with two-dimensional gel electrophoresis analysis and we, in Camilla Sjögren's laboratory, contributed with ChIP-on-chip and BrdU-IP analysis. The study was built around two hypomorphic Nse5 alleles, *nse5-ts1* and *nse5-ts2*. These alleles rendered cells temperature-sensitive, with *nse5-ts1* cells being inviable at 37°C, whereas *nse5-ts2* cells showed very slow growth under these conditions. However, the majority of the experiments were performed at their permissive temperature, 25°C, at which these mutants, under unchallenged conditions, grow as wild-type cells.

Nse5 was first shown to interact with SUMO (Smt3 in *S. cerevisiae*) by yeast-two-hybrid analysis. SUMO is a small protein that can be conjugated to target proteins and modify their functions. Nse5 also interacted with a mutated form of SUMO, which cannot be conjugated to target proteins. This result indicated that Nse5 interacts with SUMO through non-covalent interactions. Consistently, sumoylated forms of Nse5 could not be detected by SUMO pull-down experiments. Yeast-two-hybrid analysis of the two *nse5-ts* mutants showed that they did not interact with SUMO. Based on these results and the proximity of the Nse5-Nse6 subcomplex to the SUMO-ligase Mms21 within Smc5/6, the question was asked if Nse5 affected sumoylation of Smc5, which is Mms21-dependent in the presence of MMS (Zhao and Blobel, 2005). The results showed that Smc5 sumoylation was strongly reduced in both *nse5-ts* mutants.

Hypomorphic alleles of the Smc5/6 complex, including SUMO-ligase deficient alleles of Mms21, cause HU-sensitivity (Ampatzidou et al., 2006; Chen et al., 2009; Cost and Cozzarelli, 2006; Hu et al., 2005; Pebernard et al., 2006). Therefore, the two *nse5-ts* alleles

were tested for HU-sensitivity. The results showed that *nse5-ts1*, but not *nse5-ts2*, cells were HU-sensitive. Together with the previous results, these findings separate Smc5 sumoylation from the functions of Smc5/6 during HU-induced replication stress. ChIP-qPCR was then used to examine replisome stability at early firing origins in HU-treated cells. ChIP-qPCR of the polymerase epsilon subunit Pol2 was performed in a time-course experiment in cells released from G1-arrest into HU-containing medium. In wild-type and *nse5-ts2* cells, Pol2 could be detected at early, but not late, firing origins when cells commenced S-phase. However in *nse5-ts1* cells, barely any Pol2 enrichment was detected at early origins throughout the time course experiment. These results are in line with that *nse5-ts1*, but not *nse5-ts2*, are sensitive to HU-induced replication stress and shows that sumoylation of Smc5 is not required for replisome stability at stalled forks.

To further characterize the *nse5-ts1* allele, it was combined and compared with the SUMO-ligase deficient allele *mms21-11*. *nse5-ts1* mutants were shown to be more sensitive to HU than *mms21-11* mutants. Combining the two mutant alleles resulted in an increased sensitivity to HU, as compared to either single mutant. Similarly, Smc5 sumoylation levels were lower in *nse5-ts1* cells as compared to *mms21-11* cells, and barely detectable in the double mutant. ChIP-qPCR was then again used to analyze replisome stability at forks stalled by HU. In *mms21-11* cells, polymerase subunits were detected at early firing origins, similar to wild-type cells. However, *nse5-ts1 mms21-11* double mutants exhibited the same reduction of polymerase subunits detected at early firing origins as seen in *nse5-ts1* cells. This showed that although *mms21-11* cells displayed HU-sensitivity, replisome components remained at the stalled forks at wild-type levels.

In an attempt to correlate the HU-sensitivity with events taking place at the stalled forks, two-dimensional gel electrophoresis analysis of an early origin was performed. In these experiments psoralen crosslinking was used to enhance the detection of X-shaped molecules. The results showed that in the presence of HU, X-shaped molecules accumulated in *nse5-ts1*, *mms21-11* and *nse5-ts1 mms21-11* mutants at an early origin. The accumulation of X-shaped molecules was strongest in the double mutant. These X-shaped structures were dependent on Rad51, a protein that promotes the strand invasion step during homologous recombination (Krogh and Symington, 2004; Paques and Haber, 1999). This indicated that the X-shaped molecules that accumulate at replication origins in the absence of proper Nse5 and/or Mms21 functions are formed by homologous recombination.

To further characterize why *nse5-ts1* mutants displayed strong HU-sensitivity, we performed BrdU-IP-chip in the presence of HU, in the mutant and in wild-type cells. The results showed that early origins had fired in *nse5-ts1* cells similar to wild-type cells. The replication forks had also progressed similar distances away from the origins in the two strains. The fact that late origins had not fired in the mutant was a strong indication that the intra-S-phase checkpoint was intact in the mutant. However, the signal of incorporated BrdU was lower in *nse5-ts1*, than in wild-type cells. These results indicated that DNA synthesis was lower in *nse5-ts1* mutant, potentially because replisomes occasionally collapse and dissociate from DNA.

We then turned to see if Smc5/6 was recruited to the stalled forks. Smc5/6 had previously been shown to associate to HU-induced stalled forks in checkpoint mutants (Lindroos et al., 2006). We now showed, using ChIP-on-chip, that Smc5/6 was also clearly enriched around the early firing origins in HU-treated wild-type cells. However, in *nse5-ts1* cells, Smc5/6 was not detected. This result was confirmed at a few early origins using ChIP-qPCR. Using this technique, *nse5-ts2* and *mms21-11* mutants were shown not to impair the recruitment of Smc5/6 to stalled forks. Lastly, co-immunoprecipitation analysis of Nse6-Smc5 interaction, as a measure of the stability of the complex, showed that this interaction was weakened in *nse5-ts1* cells. These results indicated that Nse5 is important for the stability of Smc5/6.

In summary, the results showed that Nse5 is important for Smc5 sumoylation. However, the functional consequence of Smc5 sumoylation remains unknown since the results separated the function of Smc5/6 in HU-induced replication stress from Smc5 sumoylation. Smc5/6 was also shown to localize around replication forks stalled by HU. This localization required a stable complex and proper Nse5 function. Since HU causes fork stalling that is not site-specific, and ChIP-assays are performed on populations of cells, it is not possible, in this case, to say if Smc5/6 associated directly to the stalled forks or behind the forks, as was seen in **Paper III** where Smc5/6 accumulated behind transcription-induced paused forks. Our data suggested that Smc5/6 stabilizes stalled forks during HU-induced replication stress. This required a functional Nse5 subunit, but not the SUMO-ligase activity of Mms21. In addition, Smc5/6 prevented the accumulation of homologous recombination intermediates. This function required a functional Mms21 subunit, whose critical sumoylation target remains to be determined since Smc5 sumoylation was shown to be dispensable.

Unlike the other well-conserved subunits of Smc5/6, Nse5 and Nse6 have so far only been found in yeast. In addition, they are not conserved on the sequence level between *S. cerevisiae* and *S. pombe* (Duan et al., 2009). Nse5 and Nse6 are also essential in *S. cerevisiae* (Duan et al., 2009; Zhao and Blobel, 2005), but not in *S. pombe* (Pebernard et al., 2006). Furthermore, the Nse5-Nse6 subcomplexes bind to different places to the Smc5-Smc6 heterodimer in the two yeast species. Whereas Nse5-Nse6 in *S. cerevisiae* was found to associate to the hinge domains of Smc5-Smc6 (Duan et al., 2009), Nse5-Nse6 in *S. pombe* was found to associate to the head domains and the adjacent coil-coiled regions of the Smc5-Smc6 heterodimer (Palecek et al., 2006). Criticism could be raised against studying Nse5 in *S. cerevisiae*, since it potentially will lead to *S. cerevisiae*-specific findings. However, using these two well-established model organisms to understand the species-specific differences will potentially lead to new insights into Smc5/6 functions, applicable to other species as well. It would be interesting to see if also *S. pombe* Nse5 is important to allow the recruitment of Smc5/6 under replication stress conditions. In addition, it will be interesting to characterize the two *nse5* alleles at restrictive temperatures in unchallenged *S. cerevisiae* cells, to see if they under these conditions also affect the stability of the complex and association with chromatin. Potentially, investigations of why Smc5/6 in *S. cerevisiae*, but not *S. pombe*, requires Nse5 to promote survival will add important clues to the elusive essential function of the otherwise well-conserved complex.

PERSPECTIVES AND CONCLUDING REMARKS

The overall aim of this thesis was to investigate Smc5/6 function and chromosomal association, and how the topological status of chromosomes affects the two. We have reached this aim by revealing new functions of Smc5/6 and topoisomerases during replication (**Paper I**). Our data also suggest that Smc5/6 associates to entangled chromosomal loci, and promotes the segregation of short entangled chromosomes (**Paper II**). In the absence of cohesin function, Smc5/6 does not associate with chromosomes after replication (**Paper II**). Therefore, phenotypes found in cohesin mutants could also stem from the lack of chromatin-bound Smc5/6. In addition, we speculate that the level of topological stress on chromosomes during S-phase affects their level of entanglement (**Paper I**, **Paper II** and **Paper III**). Lastly, investigations of Nse5 during replication stress suggest that Smc5/6 maintains replication forks that have been stalled by HU treatment, and prevents aberrant homologous recombination at these forks (**Paper IV**). Our studies have revealed an intricate interplay between chromosome structure, topoisomerases and Smc5/6, in which topological structures affect SMC complexes and SMC complexes affect topological structures. Our findings allow us to speculate that topological structures are more than problems that cells need to overcome, and instead are important to link the processes of DNA replication and chromosome segregation, and thereby promote genome stability. In the sections below, this hypothesis is extended, and future challenges related to our main findings are discussed.

Potential benefits of topological structures

Topological structures, such as SCIs and positive supercoils, have mainly been considered as problems that cells need to overcome. On a speculative note, I would like to propose that these structures play important roles in the faithful segregation of chromosomes. Recent data have showed that SCIs are tightly regulated by SMC complexes. The most telling example is that cohesin maintains SCIs on plasmids until anaphase, by preventing Top2 to resolve them prematurely (Farcas et al., 2011). This revived the hypothesis of that SCIs contribute to proper sister chromatid cohesion (see below). In addition, condensin has been shown to promote Top2-dependent resolution of SCIs (Baxter et al., 2011; Charbin et al., 2014), and our data suggest that Smc5/6 helps to form them, and prevents them from becoming aberrant linkages, in the absence of Top2 (**Paper I** and **Paper II**). Our results also indicate that topological stress during replication promotes SCI formation, possibly by promoting replication fork rotation (**Paper I**, **Paper II** and **Paper III**). These results open for that

topological stress during replication is not just a problem that replication forks need to overcome, but also has the potential to contribute to proper chromosomes segregation, through SCI formation and/or sister chromatid cohesion (see below). There are a few indications in the literature of these ideas. Reducing the length of an *S. cerevisiae* chromosome below 150 kb results in that it is no longer stably transmitted (Murray et al., 1986). The authors hypothesized at the time that SCIs held chromosomes together and that such structures were not stable on chromosomes shorter than 150 kb. Subsequent analyses found that plasmids were not intertwined in metaphase cells, which showed that something other than SCIs holds counteracts the pulling forces of the spindle (Koshland and Hartwell, 1987). This was then discovered to be the cohesin complex (Guacci et al., 1997; Michaelis et al., 1997). Based on the finding of cohesin, SCIs were considered to be an unwanted byproduct of DNA replication, which could cause problems in chromosome segregation. In the light of that cohesin maintains SCIs on longer plasmids (Farcas et al., 2011), it would be interesting to revisit the former hypothesis that SCIs are needed for proper chromosome segregation of much longer linear chromosomes. Smc5/6 associate with chromosomes in a length-dependent manner (**Paper I**) and potentially marks entangled loci (**Paper II**). Therefore, it would be interesting to investigate Smc5/6 chromosomal association on chromosomes of shorter than natural length to see if it correlates with the lack of proper segregation also in Top2-proficient cells. Lastly, if SCIs have a function for proper cohesion on linear chromosomes, our data would then indicate that also the topological stress during replication (**Paper I** and **Paper II**) have positive functions in promoting fork rotation (and SCI formation) around centromeres.

FUTURE SMC CHALLENGES

Although research of SMC complexes has advanced our knowledge of chromosome dynamics substantially over the years, there are still many remaining mysteries of how the SMC complexes function. One of the most obvious ones is that the essential function of Smc5/6 still remains elusive. Hypomorphic Smc5/6 mutants showed some improved growth when homologous recombination was inhibited (Torres-Rosell et al., 2005). However, Smc5/6's function in recombination cannot alone account for Smc5/6's essential function, since homologous recombination is not required for growth in unchallenged *S. cerevisiae* cells (Krogh and Symington, 2004; Paques and Haber, 1999). Nor can Smc5/6's function in rDNA maintenance account for that unchallenged cells require the complex for viability. As mentioned before, this is due to the observation that relocalization of the rDNA to a

multicopy plasmid, from its endogenous position on chromosome 12, did not improve the growth of Smc5/6 mutants (Torres-Rosell et al., 2005). Therefore, the essential function of Smc5/6 must lie outside the maintenance of the rDNA. Similarly, the replication delay of long chromosomes seen in mutants of Smc5/6 (**Paper I**) is unlikely to be the essential function since *mms21-CH* and *top1Δ* mutants, which are viable, display a similar phenotype. Smc5/6 was recently shown to restrain Mph1 helicase function in promoting fork regression *in vitro* (Xue et al., 2014). In addition, deletion of Mph1 has been shown to restore growth, although extremely poorly, to cells lacking Smc5/6 (Chen et al., 2009). Potentially, one reason that Smc5/6 mutants fail to proliferate is that Mph1 promotes aberrant fork reversal, which causes toxic replication intermediates. It would be interesting to combine factors that potentially can alleviate the requirement of Smc5/6, such as deletion of Mph1 and inhibition of recombination. It would also be possible to engineer *S. cerevisiae* cells that do not have long chromosomes, which require Smc5/6 for their proper replication. Although Smc5/6 is essential in yeast (Lehmann et al., 1995) and mouse (Ju et al., 2013), surprisingly, this well-conserved complex is not essential in *Drosophila melanogaster* (Li et al., 2013) and *Caenorhaditis elegans* (Bickel et al., 2010). Future analyses of the reason for this difference have the potential to add important clues to the essential function of Smc5/6.

Another SMC mystery that relates to this thesis is the mechanism of sister chromatid cohesion. The dominant model is that a single cohesin ring embraces two DNA molecules from different sister chromatids (Gruber et al., 2003; Haering et al., 2002; Nasmyth and Haering, 2005). An elegant study recently succeeded with the reconstitution of cohesin's topological embracement of DNA *in vitro* (Murayama and Uhlmann, 2014). Potentially such experiments may in the future lead to answering the question if a single cohesin ring can encircle two DNA molecules. In addition, knowledge is also lacking if all, or just a subset of, cohesin complexes on chromosomes become cohesive during S-phase. Since sister chromatid cohesion is required for Smc5/6 chromosomal association, uncovering this mechanism will shed light on the regulation of Smc5/6 as well. Ideas on how cohesion establishment can be further explored are discussed in the sections below.

Distinguishing between SCIs and cohesin-mediated cohesion

Cohesin associates with chromosomes in a dynamic manner before DNA replication (Bernard et al., 2008; Gerlich et al., 2006; Lopez-Serra et al., 2013), showing that cohesin can associate with chromosomes in a non-cohesive state. Through a poorly understood mechanism

occurring during replication, sister chromatid cohesion is established, and cohesin is stabilized on chromosomes (Lopez-Serra et al., 2013). In the absence of cohesion establishment, *e.g.* in *S. cerevisiae* *eco1* mutants, sister chromatids separate as soon as they are formed, but cohesin association with these chromosomes remains unaltered (Lengronne et al., 2006). Altogether this shows that the presence of cohesin on chromosomes does not indicate whether or not sister chromatids are cohesed. Smc5/6, on the other hand, accumulates on chromosomes during replication (**Paper I**) and dissociates when sister chromatid cohesion is dissolved in anaphase (**Paper II**). In addition, Smc5/6 localizes to a subset of cohesin sites on chromosomes, and requires sister chromatid cohesion to associate with chromosomes (**Paper II**). Apart from the localization at core centromeres, Smc5/6 chromosomal association could therefore mark sites where cohesin is cohesive, *i.e.* has established cohesion. This would potentially stand as an alternative model to that Smc5/6 associates to SCIs. This alternative model would lead to the unexpected conclusion that longer chromosomes have more cohesive cohesin than shorter ones in wild-type cells. If so, the reduction in transmission stability of chromosomes shorter than 150 kb in otherwise wild-type cells (as discussed above) could be due to a reduction in cohesive cohesin, as opposed to a reduction of SCIs. Interestingly, a study suggested that enhanced replication fork pausing promote cohesion establishment (Fernius and Marston, 2009). Potentially replication forks need to slow down at specific loci (*e.g.* cohesin sites) to promote cohesion establishment. Incorporating our model proposed in **Paper II** to this idea, creates the possibility that lower topological stress on chromosomes shorter than normal length, does not slow down replication forks enough to promote proper cohesin-dependent sister chromatid cohesion establishment.

This alternative model of Smc5/6 association would also suggest that Top2 has a function in preventing excessive cohesion establishment, since Smc5/6 accumulates on chromosomes in the absence of Top2. *top2* mutants would then have more cohesive cohesin along chromosome arms, which recruits more Smc5/6. The hypothesis that replication fork slowdown promotes cohesion establishment could also be used to explain this unanticipated hypothesis, since replication termination is slowed down in *top2* mutants (Fachinetti et al., 2010). This could allow for more time for cells to establish cohesion, which would results in more cohesive cohesin (and Smc5/6) along chromosome arms.

The hallmark of cohesion establishment is the acetylation of Smc3 by Eco1 during replication (Rolef Ben-Shahar et al., 2008; Unal et al., 2008). This post-translational modification is then reversed by the histone deacetylase Hos1, after cohesin-cleavage in

anaphase (Beckouet et al., 2010). Therefore the chromosomal association of acetylated Smc3 follows that of Smc5/6. In human cells, the cohesin-associated protein Sororin is another protein that associates with chromosomes in a manner that coincides precisely with the timing of cohesion (Nishiyama et al., 2010). Sororin's chromosomal association depends on the acetylation of Smc3 (Nishiyama et al., 2010). However, this is not the case for Smc5/6 in yeast, since the complex also associates with chromosomes in *eco1 wpl1* double mutant. In this double mutant the cohesion anti-establishment function of Wpl1 is deleted, which leaves Eco1 dispensable and cohesion partially rescued, without any Smc3 acetylation (Rolef Ben-Shahar et al., 2008; Rowland et al., 2009; Sutani et al., 2009). This shows that Smc3-acetylation does not recruit Smc5/6 to cohesin sites. A future experiment that would explore if Smc5/6 association correlates with cohesive cohesin would be to perform ChIP against the acetylated form of Smc3 in wild-type and *top2* mutant cells. ChIP-seq of acetylated Smc3 has been performed successfully in human cells, which showed that acetylated Smc3 overlaps with a subset of Rad21 (the human homolog of Scc1) peaks (Deardorff et al., 2012).

A strong argument against that cohesive cohesin complexes, and not SCIs, attracts Smc5/6 to chromosomes stems from the fact that Top2 restoration causes dissociation of Smc5/6 from chromosome arms. This would then argue for the unlikely scenario that Top2 activity can remove cohesive cohesin in G2/M-arrested cells. Top2's well-established role in the resolution of SCIs therefore strongly argues that it Smc5/6 is more likely to associate with SCIs than cohesive cohesins. In addition, unpublished experiments from the Sjögren lab have failed to detect any interactions between of Smc5/6 and cohesin by mass spectrophotometry (T. Kanno and K. Jeppsson). However, these two alternative models could be united if SCIs were exclusively present at cohesive cohesin complexes. To distinguish between the hypotheses that SCI or cohesive cohesin are attracting Smc5/6 to linear chromosomes, new techniques would be needed that either has solved the formidable task of detecting SCIs on linear chromosomes, and/or allowed the creation of cohesed linear chromosomes that completely lack intertwinings between its sister chromatids.

Correlating chromosomal binding sites with function

SMC complexes have been extensively studied by ChIP analyses under various conditions. The knowledge of when and where the complexes associate to chromosomes correlates, has not only led to more detailed understanding of already known functions of SMC complexes (for example as in **Paper IV**), but also spurred investigations that found new unexpected

functions of SMC complexes (for example as in **Paper I**). In future studies attention will be turned towards understanding which specific functions the different SMC complexes perform at particular binding sites. Moving towards this goal, it is important to remember that although ChIP, especially in combination with massive parallel sequencing, is a powerful technique that allows the interrogation of a target proteins association to every position in the genome with high resolution in a single experiment, the experiments are based on populations of cells. The fact that ChIP data is a population-average of a target protein's chromatin enrichment at a particular time point opens for several scenarios of why high enrichment signal is detected at some loci, and low signal other loci. This could be due to that the protein interacts with “strong” loci in all of the cells in the population. Alternatively, it could also depend on that the target protein associates in a more stable manner at the “strong” loci, and more dynamically at “weak” loci. One step towards understanding if “weak” sites result from a more dynamic interaction of SMC complexes would be to use a recently developed ChIP assay, which allows measurements of how dynamic interactions are between the target protein and chromosomes (Poorey et al., 2013). In this assay, the experiment is repeated with different durations of formaldehyde crosslinking, and then uses mathematical models to calculate on- and off-rates of the target protein, based on the relationship between cross-linking time and ChIP-signal.

To distinguish what function an SMC complex performs at a particular site, a site-specific functional assay along with the ability to create or remove binding of the SMC complex to that locus is an ideal experimental setup. Although not a trivial task, an experiment of this kind has been performed investigating cohesin (Chang et al., 2005). In G2/M-arrested cells, a specific locus with or without cohesin binding was looped-out from the rest of the chromosome by highly efficient site-specific recombination to form circles. If this locus was cohesed, the two excised circles (one from each sister chromatid) were held together at a high rate, as monitored by GFP-targeted to this locus. Using this assay, the authors could draw conclusions of the regulation of cohesin and cohesion by silencing factors associating to this locus (Chang et al., 2005; Wu et al., 2011).

Similarly, site-specific functional analyses at a locus with or without Smc5/6 enrichment are possible based on the findings presented in **Paper III**. As mentioned before, since high convergent transcription site-specifically recruits Smc5/6, chromosome segregation of this locus could be monitored in the absence or presence of Smc5/6 enrichment, in various mutants.

A method that could be used to differentiate between binding sites of an SMC complex is to perform ChIP against specific post-translational modifications carried by subunits of the complex. This would require that the post-translational modification has been characterized and highly specific antibodies have been produced that specifically recognizes the modified form of the subunit. As mentioned above, this has been performed for the acetylated version of Smc3 in human cells (Deardorff et al., 2012). Their results showed that acetylated Smc3 overlapped with a small subset of cohesin sites. However, it remains unknown if sister chromatid cohesion is uniquely established at sites where acetylated Smc3 is present.

In addition, our understanding of how SMC complexes associate with chromosomes needs to move from a two-dimensional to a three-dimensional view. Since cohesin and condensin function to promote chromosome condensation, and Smc5/6 associates with cohesin sites, the possibility exists that two different bindings sites on the same chromosome, detected by ChIP, are created because a single SMC complex links two distant chromosomal loci intramolecularly (Figure 8).

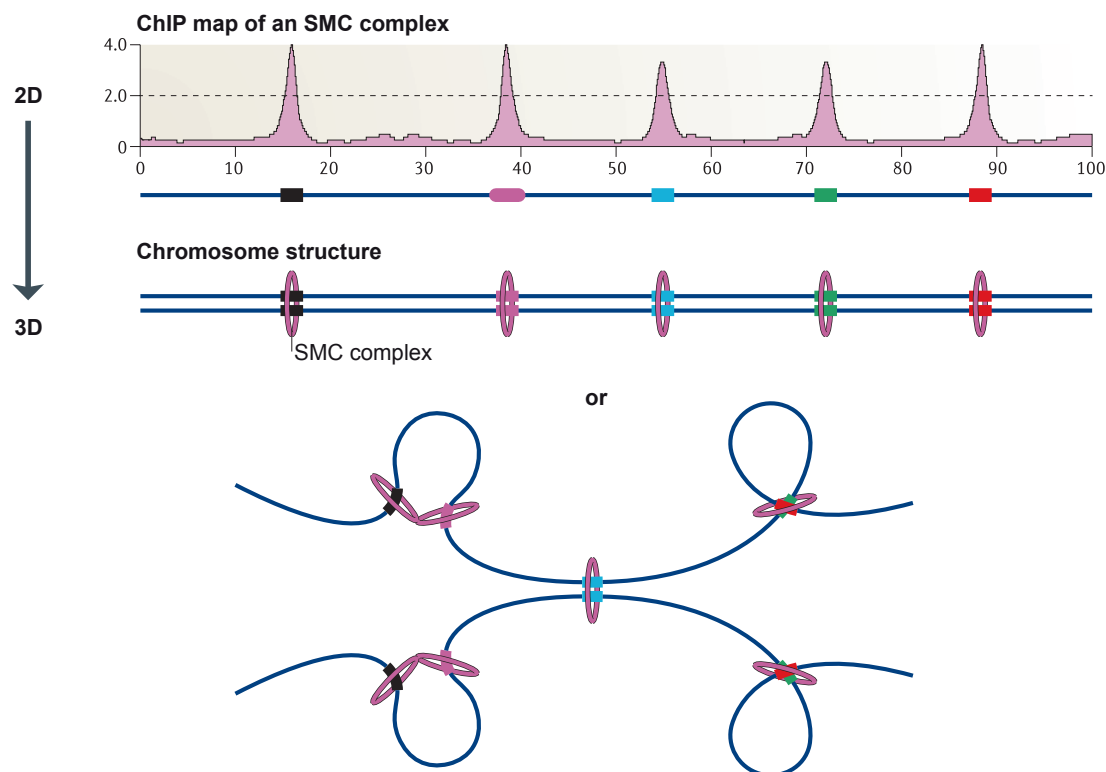


Figure 8. Potential three-dimensional conformations of SMC bound chromatin

The upper panel shows a ChIP-map of an SMC complex. Two examples of the three-dimensional chromosome structure, which the ChIP-map could represent, are displayed below. Adapted from Jeppsson et al., 2014.

A solution can be to combine ChIP data with data from chromosome conformation capture techniques (for example Hi-C). Although Hi-C techniques do not yet provide good resolution in *cis*, a recent study showed that cohesin promotes the local interactions within short regions of *S. pombe* chromosomes, to form what the authors named as “globules” (Mizuguchi et al., 2014). Not only were these “globules” cohesin-dependent, but cohesin was also found localizing at their borders. The finding that particular cohesin sites are in close proximity to each other, leads to a three-dimensional understanding of cohesin’s chromosomal association and creates the possibility that cohesin at these loci promotes condensation. An important future step towards a better three-dimensional view of genomes is to improve the resolution of Hi-C techniques, especially in *cis* (*i.e.* between loci on the same chromosome).

Analysis of replication termination

Our results indicate that SCI formation and sister chromatid cohesion promote the chromosomal association of Smc5/6 (**Paper II**). Both of these processes are also linked to replication termination. SCI formation has been suggested to occur mainly at termination sites, when the region between the two forks becomes gradually shorter. The short length of the region between the two forks might prevent topoisomerases to resolve the few remaining supercoils ahead of the forks. This would leave fork rotation as a means to reduce the topological stress ahead of the fork, in order to complete replication (Wang, 2002). Cohesion is formed during replication and is not theoretically needed before replication termination, since sister chromatids are not able to separate before (Guacci and Koshland, 2012). In addition, Smc5/6 is found specifically at cohesin sites (**Paper II**), which opens for that SCIs might be present at cohesive cohesin sites after replication has completed. These connections suggest that studies of replication termination will increase our understanding of Smc5/6.

However, replication termination is difficult to analyze since it does not occur at specific sites but instead in wider regions, *i.e.* termination regions or fork merger zones (Fachinetti et al., 2010; McGuffee et al., 2013). In a population of cells it is also difficult to temporally synchronize these events. In addition, replication termination intermediates are short-lived due to their rapid resolution, which results in that converging forks are difficult to analyze by two-dimensional gel electrophoresis analysis. One idea to allow more detailed studies of replication termination is to create termination “hot spots” at which all cells in a population terminate replication in temporally synchronous manner. Pausing replication forks

long enough should create site-specific replication termination, when the converging fork arrives. Potentially, further development of the experimental setup described in **Paper III** can achieve this task.

The recent finding that a specific post-translational modification of terminated replisomes promotes their disassembly (Maric et al., 2014; Moreno et al., 2014), enables the use of specific antibodies against the post-translationally modified version of the protein in ChIP-assays to further investigate replication termination.

Visualization of topological structures on linear chromosomes

The visualization of topological structures, such as supercoils and SCIs, present on linear chromosomes *in vivo* poses a significant challenge for the future. An initial attempt have been made to take on the daunting task of measuring levels of topological stress on linear chromosomes *in vivo*, using a assay based on psoralen crosslinking (Bermudez et al., 2010). Psoralen intercalates DNA, and the more unwound the DNA helix is the more psoralen will intercalate. Specifically purifying psoralen crosslinked DNA therefore allows analysis of which regions have a higher degree of negative supercoiling. The results suggested that domains with different degrees of topological stress exist on *S. cerevisiae* chromosomes, and that regions close to chromosome ends contain less topological stress than the rest of the genome. The authors speculated that coupling this assay to massive parallel sequencing, instead of microarray hybridization would improve the resolution, and potentially lead to more detailed findings (Bermudez et al., 2010).

To date there is no technique available to directly detect SCIs present on linear chromosomes *in vivo*. Since SCIs are just two DNA double helices that are wrapped around each other, these structures have the potential to be highly mobile, especially when chromosomes are extracted from cells for analysis. Conclusions of SCI dynamics on linear chromosomes, including ours, are therefore based on indirect observations. For example, chromosome length-dependent missegregation in the absence of Top2 activity ((Spell and Holm, 1994) and **Paper II**) is concluded to depend on the failure of resolving SCIs, without directly visualizing SCIs. An attempt has been made to analyze SCIs on human metaphase chromosomes *ex vivo* (Bauer et al., 2012). In this study chromosomes were deproteinized and then Top2-sensitive DNA linkages could be visualized between the sister chromatids. However, the results from this assay does not provide evidence if the DNA linkages are truly SCIs, or potentially due to unfinished replication, nor does it provide information of which

chromosomal loci are entangled. This is also true for assays looking at DNA-linkages, *e.g.* ultra-fine bridges, between segregating chromosomes *in vivo* (Liu et al., 2014; Oliveira et al., 2010; Wang et al., 2010).

Our data showed site-specific alterations (Smc5/6 accumulation) on chromosomes in the absence of Top2, conditions which cause reporter plasmids to become highly intertwined and linear chromosomes to missegregate in a length-dependent manner (**Paper II**). The accumulation of Smc5/6 follows in a precise manner what would be expected for SCIs. Based on this, we proposed that Smc5/6 associates to SCIs (**Paper II**). These findings highlight specific loci and conditions that should be focused on when assays are being developed to directly visualize SCIs.

First, a step could be taken to investigate if Top2 uses its well-characterized catalytic function at these sites. Our experimental setup of restoring Top2 activity after replication, which causes Smc5/6 dissociation from chromosome arms (**Paper II**), can be extended to directly map the sites of Top2 catalytic activity. This can be achieved by treating cells with the Top2 poison etoposide before Top2 activity is restored. Etoposide prevents Top2 to religate the created double strand break (Pommier et al., 2010), resulting in that Top2 remains covalently bound to the DNA cut site. Thereafter Top2-DNA complexes can be purified and Top2 cut sites can be mapped with nucleotide resolution (Haffner et al., 2010; Mirault et al., 2006). This experiment would pinpoint the loci of Top2 catalytic action, and if they coincide with Smc5/6 chromosomal binding would further strengthen the idea that SCIs are present at those sites. However, the exact structure that Top2 resolves at these loci would still remain unknown. Instead, applying an assay to site-specifically recombine out circles from chromosomal regions bound by Smc5/6 has the potential to confine whatever topological structures that were present at Smc5/6 binding sites *in vivo*. Identification of which structures they are could be performed by subsequent gel electrophoresis analysis. The visualization of SCIs on linear chromosomes will not be straightforward, but the successful accomplishment of this task will be highly rewarding. It will not only provide valuable knowledge of chromosome structure, it will also help to answer if SCIs contribute to sister chromatid cohesion, and add important knowledge to the regulation of Smc5/6.

FINAL REMARKS

The discovery of topoisomerases solved the conundrum about how the DNA helix could be replicated and segregated, without the problems of DNA tangling (Wang, 2002). Later, studies of SMC complexes provided a fundamentally new understanding of how chromosomes are organized to promote correct segregation. Since cancerous cells are often aneuploid, investigating the processes by which chromosomes are replicated and segregated can lead to more detailed knowledge of how cells become aneuploid. This emphasizes the importance of further studies of SMC complexes and topoisomerases. In addition, mutations in subunits of SMC complexes and associated proteins have been found in several genetic disorders (Deardorff et al., 2012; Deardorff et al., 2007; Gordillo et al., 2008; Krantz et al., 2004; Musio et al., 2006; Payne et al., 2014; Tonkin et al., 2004; Vega et al., 2005). This highlights the importance of elucidating the mechanisms of their functions to learn more of the etiology of these diseases. The mechanisms of topoisomerases are already well characterized, and they are also the targets of several anti-cancer agents (Pommier et al., 2010). However, these drugs suffer from the targeting of healthy cells. Potentially, further studies of the regulation of topoisomerases can lead to improving the specificity of anti-cancer agents.

The studies presented in this thesis reveal new functions of Smc5/6 and topoisomerases during DNA replication, and of Smc5/6 in chromosome segregation. For example, our results add more specific knowledge about the outcomes of Top2 inhibition during different stages of the cell cycle (**Paper II**). Another important conclusion of this thesis is that Smc5/6 associates with chromosomes only at times and positions where sister chromatids are in close proximity to each other, *e.g.* at replication forks (**Paper III** and **Paper IV**) and at cohesin sites where chromosomes might be entangled (**Paper II**). Since our results (**Paper I**, **Paper II** and **Paper IV**) and previous studies (Copsey et al., 2013; Gallego-Paez et al., 2014; Lilienthal et al., 2013; Torres-Rosell et al., 2005; Xaver et al., 2013) have shown that aberrant linkages between sister chromatids arise in Smc5/6 mutants under a variety of conditions, further investigations of which DNA transactions that occur at the Smc5/6 binding sites have the potential to shed light on the mechanism of Smc5/6 and its elusive, yet essential function. Speculatively, our results might also enable future detailed studies of entanglements on linear chromosomes. Lastly, our findings suggest that topological stress is dealt with on a chromosomal scale (**Paper I** and **Paper II**), and that such stress during replication can influence chromosome segregation.

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